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# Alterations at the blood-brain barrier and brain parenchyma along brain metastasization of breast cancer

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## Abstract

Despite the restricted permeability of the blood-brain barrier (BBB), the brain is a privileged organ regarding the appearance of metastases, particularly from breast cancer. Patients with brain metastases from breast cancer have a severe prognosis, rendering this issue a serious oncologic problem that deserves further attention. Therefore, additional studies are required to establish when breast cancer cells cross the brain endothelium and what are the routes used for the transendothelial migration, to understand what is their precise phenotype along the processes of transmigration and establishment of brain metastases, to determine the alterations occurring in brain endothelium, to study how endothelial cells communicate with malignant ones to promote the attraction of malignant cells into the brain vasculature and tumour-associated vascular development. Based on this, we aimed at establishing the temporal profile of breast cancer metastasization to the brain and characterize the metastasizing cells phenotype, as well as, to investigate the vascular events and BBB properties along the process of metastasization to this target organ. In addition, we aimed to assess signalling mechanisms involved in attraction of carcinoma cells into the brain and proliferation in the nervous tissue. To establish the temporal evolution of the players involved in such processes, we used cerebella, cranial hippocampi, and striata of female mice inoculated with 4T1 breast cancer cells sacrificed at 5 hours, 3 days, 7 days or 10 days, and of female mice injected with vehicle (control) sacrificed at 5 hours. Our results showed the presence of brain metastasis of breast cancer at 7-days after inoculation, which increased thereafter. The malignant cells crossed the BBB as mesenchymal cells and, once inside the brain, these cells underwent a complete or partial mesenchymal-epithelial transition to acquire the epithelial characteristics that allow the establishment of new tumours. In addition, the process of brain metastasization of BC contributed to the downregulation of the tight junction protein claudin-5 of brain microvascular endothelial cells, as well as to the entrance of the blood-borne component thrombin in brain parenchyma. On the other hand, hypervascularization in cranial hippocampus appeared to be associated to the process of brain colonization by breast cancer cells. Regarding the role of platelet-derived growth factor B signalling along the process of brain metastasization, we found that this growth factor was expressed by tumour cells and its expression increased during the formation of brain metastasis. Interestingly, a continuous entrance of cysteine-X amino acid-cysteine receptor 4 (CXCR4)-positive cells into the brain parenchyma appeared to occur

along the process of brain metastasization of breast cancer. In sum, this study contributes to clarify the time-course and interdependence of the signalling events, BBB breach and phenotypic transition of malignant cells along endothelial transposition and brain metastases establishment by breast cancer cells. Moreover, the demonstration of early cellular and molecular events points to novel targets for modulation in order to prevent metastasis formation and development.

*Keywords*: Blood-brain barrier; brain metastasis; breast cancer; phenotypic transition; transendothelial migration.

## Resumo

Apesar da barreira hematoencefálica (BHE) ter uma permeabilidade restrita, o cérebro é um órgão preferencialmente afetado pelo aparecimento de metástases, particularmente de cancro mama. Pacientes com metástases cerebrais provenientes do cancro da mama têm um prognóstico severo, tornando a metastização num sério problema oncológico que merece toda a atenção. Por este motivo, novos estudos são necessários para estabelecer quando é que as células cancerígenas da mama atravessam o endotélio cerebral e quais são as vias que utilizam para migrarem através do endotélio, para se perceber qual o fenótipo que têm ao longo dos processos de migração para dentro do encéfalo e durante o estabelecimento de metástases, para determinar as alterações que ocorrem no endotélio cerebral, para estudar como as células endoteliais comunicam com as células malignas para promover a atracão das células cancerígenas da mama para a vasculatura do encéfalo e o desenvolvimento vascular associado ao tumor. Com base nisto, tivemos com objetivos estabelecer o perfil temporal da metastização do cancro da mama para o encéfalo e caracterizar o fenótipo destas mesmas células, assim como, estudar as alterações vasculares e as propriedades da BHE ao longo do processo de metastização para este órgão secundário. Para além disso, também pretendíamos avaliar os mecanismos de sinalização envolvidos na atracão das células tumorais para o encéfalo e na proliferação no tecido nervoso. Para estabelecer a evolução temporal dos intervenientes envolvidos em tais processos, utilizámos cerebelos, hipocampos craniais e estriados de ratinhos fêmea inoculados com células cancerígenas da mama 4T1 sacrificados às 5 horas, 3 dias, 7 dias, ou 10 dias, e de ratinhos fêmea injetados com veículo (controlo) sacrificados às 5 horas. Os nossos resultados mostraram a presença de metástases cerebrais do cancro mama 7 dias após a inoculação, aumentando ao longo do tempo. As células malignas atravessaram a BHE como células mesenquimais e, uma vez dentro do encéfalo, estas células sofreram uma transição completa ou parcial de fenótipo mesenquimal para epitelial para adquirirem as características epiteliais necessárias para o estabelecimento de novos tumores no encéfalo. Além disso, o processo de metastização cerebral do cancro da mama contribuiu para a diminuição da expressão da proteína das junções de oclusão claudina-5 nas células endoteliais microvasculares cerebrais, assim como para a entrada do componente sanguíneo trombina no parênquima cerebral. Por outro lado, o aumento de vascularização no hipocampo cranial aparentou estar associado ao processo de colonização do encéfalo pelas células cancerígenas da mama.

Relativamente ao papel da sinalização do fator de crescimento B derivado de plaquetas ao longo do processo de metastização cerebral, descobrimos que as células tumorais expressavam este fator de crescimento e que a sua expressão aumentou durante a formação de metástases no encéfalo. Curiosamente, a entrada contínua de células que expressam o recetor CXCR4 para dentro do parênquima cerebral aparentou ocorrer ao longo do processo de metastização cerebral do cancro da mama. Deste modo, este estudo contribui para clarificar o curso temporal e a interdependência de vias de sinalização, a quebra da BHE e a transição fenotípica das células malignas ao longo na transposição do endotélio e estabelecimento de metástases cerebrais pelas células cancerígenas da mama. Além disso, a demonstração dos eventos celulares e moleculares iniciais aponta para novos alvos para modulação de modo a prevenir a formação e desenvolvimento de metástases.

*Palavras-chave*: Barreira hematoencefálica; cancro da mama; metástase cerebral; migração transendotelial; transição fenotípica.

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## Abbreviations

| | |
|---|---|
| **12(S)-HETE** | 12(S)-hydroxyeicosatetraenoic acid |
| **ADAM** | A disintegrin and metalloproteinase |
| **AJ** | Adherens junction |
| **AJCC** | American Joint Committee on Cancer |
| **BBB** | Blood-brain barrier |
| **BC** | Breast cancer |
| **BCC** | Breast cancer cell |
| **BHE** | Barreira hematoencefálica |
| **BM** | Basement membrane |
| **BMVEC** | Brain microvascular endothelial cell |
| **BSA** | Bovine serum albumin |
| **bTNM** | Biologic tumour, node, and metastasis |
| **CA** | California |
| **CD** | Cluster of differentiation |
| **CK** | Cytokeratin |
| **CNS** | Central nervous system |
| **CTC** | Circulating tumour cell |
| **Cx** | Connexin |
| **CXCL12** | Cysteine-X amino acid-cysteine ligand 12 |
| **CXCR4** | Cysteine-X amino acid-cysteine receptor 4 |
| **DAB** | 3,3'-diaminobenzidine tetrahydrochloride |
| **DAPI** | 4',6-diamidino-2-phenylindole |
| **EC** | Endothelial cell |
| **E-cadherin** | Epithelial cadherin |
| **ECM** | Extracellular matrix |
| **EGF** | Epidermal growth factor |
| **EMT** | Epithelial-mesenchymal transition |
| **EndMT** | Endothelial-mesenchymal transition |
| **ER** | Oestrogen receptor |
| **E-selectin** | Endothelial selectin |
| **FCT** | Fundação para a Ciência e Tecnologia |
| **GABA** | γ-aminobutyric acid |
| **GJ** | Gap junction |
| **H&E** | Haematoxylin and eosin |
| **HER2** | Human epidermal growth factor receptor 2 |
| **HGFR** | Hepatocyte growth factor receptor |
| **HGF/SF** | Hepatocyte growth factor/scatter factor |
| **HRP** | Horseradish peroxidase |
| **ICAM-1** | Intercellular adhesion molecule-1 |

| | |
|---|---|
| **IF** | Immunofluorescence |
| **IHC** | Immunohistochemistry |
| **JAM** | Junctional adhesion molecule |
| **Lhx2** | LIM homeobox gene 2 |
| **MA** | Massachusetts |
| **Mc** | Monoclonal |
| **MET** | Mesenchymal-epithelial transition |
| **MLC** | Myosin light chain |
| **MLCK** | Myosin light chain kinase |
| **MMP** | Matrix metalloproteinase |
| **MO** | Missouri |
| **MUC1** | Mucin 1 |
| **MW** | Microwave |
| **NA** | Not applicable |
| **N-cadherin** | Neuronal cadherin |
| **NKFIH** | National Research, Development and Innovation |
| **PBS** | Phosphate-buffered saline |
| **Pc** | Polyclonal |
| **PDGF** | Platelet-derived growth factor |
| **PDGFR-β** | Platelet-derived growth factor receptor beta |
| **PECAM-1** | Platelet endothelial cell adhesion molecule-1 |
| **PR** | Progesterone receptor |
| **PSGL-1** | Platelet selectin glycoprotein ligand-1 |
| **OTKA** | Hungarian Scientific Research Fund |
| **ROS** | Reactive oxygen species |
| **SEM** | Standard error of mean |
| **sLe$^x$** | Sialyl Lewis x |
| **Snail** | Zinc finger protein snail 1 |
| **TEM** | Transendothelial migration |
| **TGF-β** | Transforming growth factor beta |
| **TJ** | Tight junction |
| **TNM** | Tumour, node, and metastasis |
| **TX** | Texas |
| **UK** | United Kingdom |
| **USA** | United States of America |
| **VCAM-1** | Vascular cell adhesion molecule-1 |
| **VE** | Vascular endothelial |
| **VEGF** | Vascular endothelial growth factor |
| **ZEB** | Zinc finger E-box-binding homeobox |
| **ZO** | Zonula occludens |

# Chapter I

## Brain metastasization of breast cancer

The work presented in this chapter corresponds to the following manuscript submitted for publication:

Brain metastasization of breast cancer
Custódio-Santos T, Videira M, Brito MA

## Abstract

Breast cancer can lead to formation of metastases in distant organs, such as the brain, which is commonly associated with diminished quality of life. In addition, central nervous system metastases have been reported in 12-31% of breast cancer patients, and the incidence is increasing with the improvement of primary tumours treatment by therapeutic agents that do not cross the blood-brain barrier, thus rendering the brain a vulnerable organ. Moreover, the survival of patients with breast cancer metastatic to the central nervous system is generally poor, with reports of a 1-year survival rate of 20%. Therefore, a better knowledge of the determinants of brain metastasization is essential for the identification of patients at risk of brain metastases formation, and for the development of novel preventive therapeutic strategies. Here, we summarize and discuss the current data about the multistep process of brain metastasization, ranging from the output of cancer cells from the primary tumour to their colonization in the brain, which involves the epithelial-mesenchymal transition that facilitates the invasion of mammary tissue, the intravasation into circulation, the survival through the vasculature that allows the arrest of the most metastatic cancer cells at brain microvasculature, and the extravasation towards this secondary organ. The several phases of extravasation are also dissected, namely the rolling, adhesion, and transendothelial migration across brain microvascular endothelial cells, and the transcellular passage pathways are addressed. Furthermore, the proliferation of metastatic cancer cells, and their colonization in the brain, the change in malignant cells phenotype via mesenchymal-epithelial transition, and the importance of the microenvironment in the formation of brain metastases are inspected. Finally, the role of angiogenesis along the process of brain metastasization is also address, playing a crucial role not only in primary tumour growth, but also in spread and proliferation of cancer cells. Such detailed cellular and molecular characterization of brain metastasization process contributes to an in-depth understanding of the malignant behaviour.

*Keywords*: Brain metastasis; breast cancer; epithelial-mesenchymal transition; transendothelial migration; tumour microenvironment.

## Resumo

O cancro da mama pode levar à formação de metástases em órgãos distantes, como por exemplo o cérebro, e esta formação de tumores secundários está normalmente associada à diminuição da qualidade de vida dos pacientes. Para além disso, metástases no sistema nervoso central têm sido reportadas em 12-31% dos pacientes e a incidência está a aumentar com o melhoramento do tratamento dos tumores primários através de agentes terapêuticos que não atravessam a barreira hematoencefálica, tornando assim o cérebro um órgão vulnerável à formação de tumores secundários. A taxa de sobrevivência de pacientes com cancro da mama metastático para o sistema nervoso central é geralmente reduzida, com estudos a reportarem uma taxa de sobrevivência de um ano de 20%. Portanto, um melhor conhecimento dos intervenientes envolvidos na metastização cerebral é essencial para a identificação de pacientes em riscos de desenvolverem metástases cerebrais e para o desenvolvimento de novas estratégias terapêuticas preventivas. Deste modo, nós sumariamos e discutimos a informação atual acerca do processo de metastização do cérebro que compreende um conjunto de passos desde a saída das células cancerígenas do tumor primário até à sua colonização no cérebro, envolvendo a transição epitelial-mesenquimal que facilita a invasão do tecido mamário, a entrada no sistema circulatório, a sobrevivência através da vasculatura que permite a chegada das células cancerígenas com maior potencial metastático à micro-vasculatura do cérebro, e a extravasação para dentro deste órgão secundário. As três fases da extravasação também são examinadas, nomeadamente o rolamento, a adesão e a migração por entre as células endoteliais micro-vasculares do cérebro, e as vias da migração trans-celular são abordadas. Para além disso, a proliferação das células cancerígenas metastáticas e a sua colonização no cérebro, a mudança de fenótipo por parte das células malignas através da transição mesenquimal-epitelial e a importância do micro-ambiente na formação de metástases cerebrais são discutidas. Por fim, o papel da angiogénese ao longo do processo de metastização do cérebro também é abordado, tendo este processo um papel crucial não só no crescimento do tumor primário, como também na disseminação e proliferação das células cancerígenas. Assim, a caracterização celular e molecular detalhada do processo de metastização cerebral contribui para uma compreensão cada vez mais aprofundada do comportamento de uma célula maligna.


*Palavras-chave*: Cancro da mama; metástase cerebral; microambiente tumoral; migração transendotelial; transição epitelial-mesenquimal.

## 1. Breast cancer

Breast cancer (BC) is a malignant tumour that usually starts in the epithelial cells of the mammary ducts, which are structures responsible for drainage of milk from the lobules secretory acini to the nipple during lactation (Videira et al. 2014). It is considered the most frequently diagnosed cancer in women, with estimated 1.7 million new cases worldwide and nearly 521,900 related deaths in 2012 (Torre et al. 2015). The early detection of this cancer through a mammography screening increases the chances for successful treatment and consequently decreases the mortality from BC (Heinävaara et al. 2016). For early-stage BC, the typical treatment procedure involves either mastectomy (total removal of the breast) or lumpectomy (removal of breast tumour and some of the normal surrounding tissue followed by radiation therapy) plus adjuvant treatment (Zujewski 2016). Early surgical intervention has made an impact in preventing the recurrence of BC. However, this solid cancer is not always timely diagnosed, and in more aggressive and advanced stages the recurrence at distant organs of the body is overwhelming.

All cancers are classified at diagnosis due to its importance for prognosis and responsiveness to therapy. The most widely used BC classification is the tumour, node, and metastasis (TNM) staging system that is based on tumour size (T), regional nodal involvement (N), and distant metastasis (M), and divides BC in four stages (Whitman et al. 2006), as summarized in Table 1. Another classification considers non-anatomical factors such as biomarkers, and distributes BC patients according to the expression of receptors in three groups: hormone receptor-positive when patient presents either oestrogen receptor (ER) or progesterone receptor (PR); human epidermal growth factor receptor 2 (HER2)-positive when HER2 is overexpressed; and triple-negative when the patient is hormone receptors-negative and HER2-negative (Mouttet et al. 2016). The hormone receptors and HER2 are cell surface receptors present in normal breast cell, and their cross-talk results in a positive feedback to cell cycle, survival, and proliferation (Chung et al. 2002, Caldon 2014). However, in BC cells (BCCs), the overexpression of HER2 together with DNA damage induced by high levels of oestrogen lead to deregulated proliferation and growth of these cells, contributing to the initiation and progression of BC. Among the three groups, the triple-negative phenotype is associated with worse survival as compared with patients with the expression of the receptors (Ovcaricek et al. 2011). So, understanding of each group of patients at a molecular level allows not only

**Table 1** Tumour, node, and metastasis (TNM) staging according to the American Joint Committee on Cancer (AJCC) staging system.

| Cancer stage | Tumour size (T) | Regional nodal involvement (N) | Distant metastasis (M) | Description |
|---|---|---|---|---|
| **Stage I** | T1 | N0 | M0 | Lesions measure 2 cm or less without metastases |
| **Stage IIA** | T0 or T1 | N1 | M0 | Lesions measure 2 cm or less with involvement of the axillary lymph node |
| | T2 | N0 | M0 | Lesions measure from 2 cm to 5 cm without involvement of the axillary lymph nodes |
| **Stage IIB** | T2 | N1 | M0 | Lesions measure from 2 cm to 5 cm with involvement of the axillary lymph node |
| | T3 | N0 | M0 | Lesions measure more than 5 cm |
| **Stage IIIA** | T0, T1 or T2 | N2 | M0 | Lesions of any size with metastases in axillary lymph nodes (absence of clinically evident axillary lymph node metastases) |
| | T3 | N1 or N2 | M0 | Lesions measure more than 5 cm with or without metastases in axillary lymph nodes |
| **Stage IIIB** | T4 | N0, N1 or N2 | M0 | Tumour of any size which directly extend to the chest wall, skin or lymph nodes |
| **Stage IIIC** | Any T | N3 | M0 | Tumour of any size with involvement of the axillary and/or internal mammary lymph nodes |
| **Stage IV** | Any T | Any N | M1 | Presence of distant metastases |

deciding the most effective treatment for each patient, but also to study and develop more specific therapeutic drugs. Since these two classification systems are routinely used in clinical practice, Bagaria et al. proposed the biologic TNM (bTNM) as a better prognostic indicator (Bagaria et al. 2014). The bTNM incorporates the biomarker profile, defined solely by the triple-negative phenotype, in the TNM staging system, whereby the stage is first determined by TNM, and the addition of the triple-negative phenotype upstages the cancer to the next stage.

The development of primary tumour of breast is a step-wise process that stars with a proliferative growth of epithelial cells in a mammary duct, and culminates in invasion of surrounding tissue, as explained below. After the initial growth of cells, tumour-

associated angiogenesis must occur if a malignant mass is to exceed 1 mm in diameter (Fidler 2002). The association between angiogenesis and cancer progression was first described by Judah Folkman et al., who stated that tumour growing was directly dependent of blood vessel network development (Folkman et al. 1971). Angiogenesis is stimulated when tumour tissues require nutrients and oxygen, being essential for cell viability. In fact, the absence of vascular support can cause the tumour to become necrotic or even apoptotic (Nishida et al. 2006). Angiogenesis involves highly regulated paracrine signalling between vascular growth factors released by cancer cells and host cells, and their respective receptors expressed on endothelial cells (ECs) of preexisting vessels. Among the proangiogenic factors expressed by BCCs are vascular endothelial growth factors (VEGFs) and angiopoietins, which can be induced through hypoxia inducible factor 1α and integrins (Felcht et al. 2012, Raja et al. 2014). On the other hand, ECs begin to release specific proteases, such as matrix metalloproteinases (MMPs), to degrade the basement membrane (BM) and extracellular matrix (ECM) that surround a nearby capillary, thereby facilitating their migration into the malignant mass (Papetti and Herman 2002). After ECs proliferation, the newly formed capillary is generally leaky due to the weak cell-cell junctions between ECs and the vulnerable reconstituted BM that surrounds these cells (Shenoy and Lu 2014). Therefore, angiogenesis plays an important role not only in BC growth and progression in primary tumour site, but also during the metastatic stage of BC. Thence, one of the therapies for solid cancers may involve the use of angiogenesis inhibitors, which would consequently block angiogenesis and tumour cell growth and may prevent metastasization to distant organs (Brave et al. 2011).

According to the TNM staging system, when the cancer reaches its most advanced or metastatic stage, tumour cells have the ability to spread and form new tumours in distant visceral organs such as lungs, liver, and brain, and/or in non-visceral organs that include bone, and skin (Berman et al. 2013). The arrest and growth of malignant cells in 'target organs' present a preferential distribution and location, a process called organotropism (Lu and Kang 2009). The organotropism depends on the following factors: the receptor status of BCCs; the circulatory pattern, despite the fact that the most frequently organs metastasized by BCCs do not have an immediate direct vascular connection with the primary tissue; genetic signatures present in tumour cells that orchestrate and control the metastatic tropism; and the microenvironment of the organ that will be metastasized (Minn et al. 2005, Spano and Zollo 2012, St Romain et al. 2012). Regarding BC, this cancer represents the second most frequent cause of central nervous

system (CNS) metastases, after lung cancer, with metastases occurring in 12-31% of patients (Vuong et al. 2011, Saha et al. 2013).

Brain metastasis is commonly associated with poor prognosis and diminished quality of life, being normally a catastrophic life-threat outcome for patients with solid cancers, such as BC (Cruz-Muñoz and Kerbel 2011, Jaboin et al. 2013). In fact, the 1-year survival rate of patients with BC metastatic to the CNS was reported as only 20% (Altundag et al. 2007). Moreover, there are no targeted therapies specific for this secondary tumour formation, and it is expected that the incidence of brain metastasis continues to increase (Clayton et al. 2004, Steeg et al. 2011). In addition, the incidence of brain metastasis from the primary breast tumour also increases among patients who received chemotherapy or targeted molecular therapeutic such as trastuzumab (Yau et al. 2006, Tonyali et al. 2016). The causes of this increased incidence of brain metastases are unknown, despite the several theories that have been posited. Since the treatment of BC improves the patient's quality of life and survival, the probability of cancer progression and hence formation of metastases, particularly in the brain, are also enhanced. On the other hand, the brain represents a 'sanctuary' organ at risk for disease relapse due to the presence of the blood-brain barrier (BBB), a highly impermeable structure that prevents CNS penetration of most of the conventional and new chemotherapeutic agents (Tonyali et al. 2016). Moreover, glial cells that compose the cerebral microenvironment have a profound impact on the efficacy of chemotherapy by upregulating drug resistance, antiapoptosis, and survival genes in cancer cells, thus rendering malignant cells resistant to therapy (Lee et al. 2016). So, both vascular ECs and brain resident cells protect BCCs from chemotherapy. Another hypothesis postulates that the incidence of brain metastases only seems to have increased since their detection is facilitated by the increased use of refined imaging and the greater attention paid to neurological signs or symptoms (Fink and Fink 2013). Therefore, a better knowledge of the determinants of brain metastasization is essential for the identification of patients at risk of formation of secondary tumour and for the development of novel therapeutic approaches able to prevent such effect from occurring and, thus, to improve the clinical outcomes. In this review, we present the processes that occur during the course of BCCs from the primary tumour site to brain. Furthermore, we explore the still intriguing and unclear mechanisms by which these cells are able to cross the BBB and form brain metastasis, which may constitute potential therapeutic targets.

## 2. From primary tumour to secondary organ

Metastasization is a multistep process ranging from the output of cancer cells from the primary tumour to their colonization in the 'target organ' (Nguyen et al. 2009). Among these steps are epithelial-mesenchymal transition (EMT) that facilitates invasion of mammary tissue and intravasation into the circulatory system, survival through the vasculature, arrest at distant organ sites, extravasation within the secondary organ, and finally proliferation and formation of metastasis. When the secondary organ is the brain, the extravasation of BCCs requires the interaction between the tumour cell and the brain microvascular endothelium that forms the BBB (Wilhelm et al. 2014). Once inside the brain, the cells that compose the microenvironment of this secondary organ play a key role in promoting proliferation and colonization by metastatic cells, providing crucial support for cell survival and tumour growth (Lorger 2012). To complete the invasive-metastatic cascade, BCCs reacquire their epithelial phenotype via mesenchymal-epithelial transition (MET), and form well-established brain metastases (Gunasinghe et al. 2012). Identifying and understanding all the mechanisms behind the BC metastasization into the brain may lead to limiting tumour progression, making all steps of the metastatic process potential targets for therapeutic intervention.

Mammary ducts are lined by columnar or cuboidal epithelial cells, surrounded by a discontinuous layer of contractile myoepithelial cells, encircled by the BM, and embedded by the ECM (Muschler and Streuli 2010, Owens et al. 2013) that separates epithelial and stromal compartments (Fig. 1). So, the morphogenesis and architecture of normal mammary epithelium are ensured by cell-cell and cell-BM interactions. Epithelial cells establish interactions with neighbouring cells through intercellular junctions that include tight junctions (TJs), adherens junctions (AJs), desmosomes, and gap junctions (GJs). TJs are located at the apical membrane of epithelial cells and their functions are the restraining of paracellular transport, and establishment and maintenance of apical-basal epithelial polarity (Giepmans and van Ijzendoorn 2009, Owens et al. 2013). These junctions are formed by transmembrane proteins occludin, claudins, and junctional adhesion molecules, and the cytosolic proteins of the zonula occludens (ZO) family that are responsible for the attachment to the cytoskeleton protein actin. AJs are located subjacent to TJs, and have a crucial role in providing adherent strength and attaching the actin cytoskeleton to the plasma membrane (Giepmans and van Ijzendoorn 2009, Owens et al. 2013). These junctions are formed by the transmembrane cadherins, namely

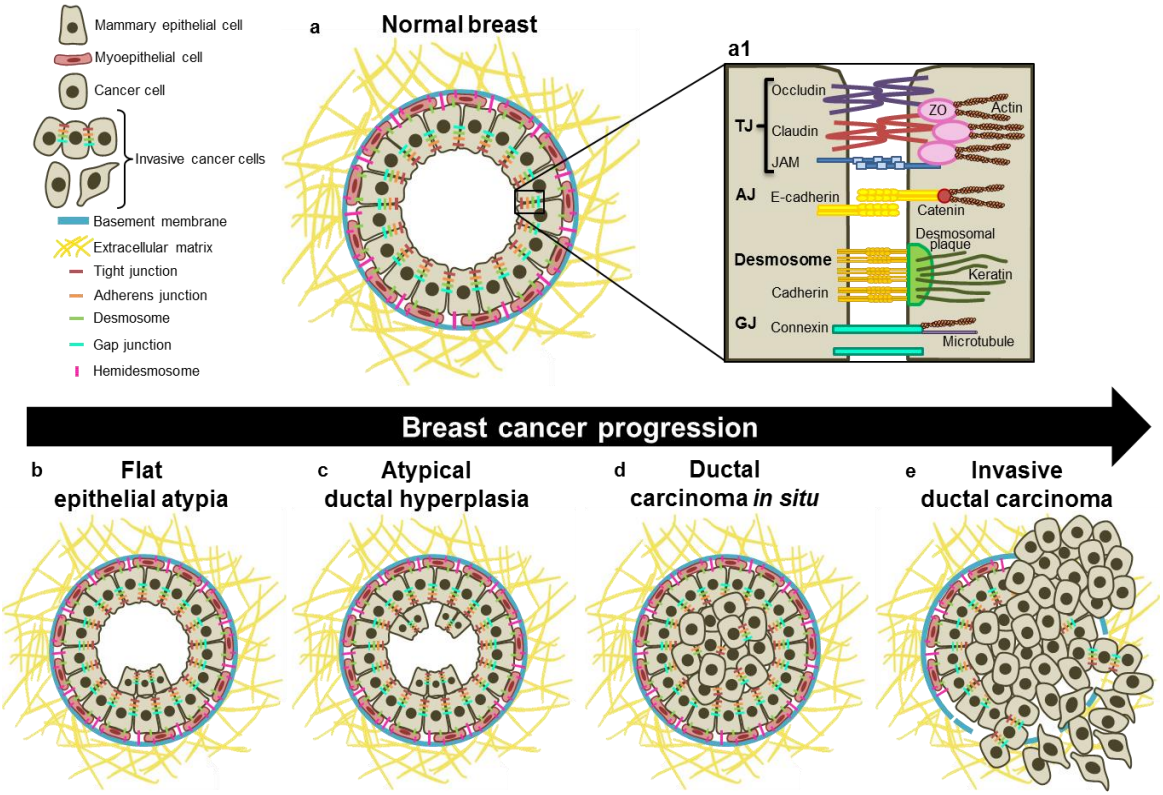


**Fig. 1** Schematic representation of the mammary duct and of the alterations occurring during the progression of breast cancer (BC). In normal breast, the mammary duct is lined by polarized epithelial cells that are surrounded by a discontinuous layer of myoepithelial cells, and encircled by a basement membrane (BM); mammary epithelial cells establish intercellular junctions between neighbouring cells and with myoepithelial cells, and attach to the BM through hemidesmosomes (a); the intercellular junctions are composed by tight junction (TJ), adherens junction (AJ), desmosome, and gap junction (GJ), and each junction possesses a specific composition and assembly (a1). The duct is embedded in the extracellular matrix of the mammary parenchyma. The development of breast primary tumour occurs through a step-wise progression from benign flat epithelial atypia to atypical ductal hyperplasia, evolves into malignant ductal carcinoma *in situ*, and finally to invasive ductal carcinoma; both flat epithelial atypia and atypical ductal hyperplasia are characterized by intraductal proliferation of epithelial cells, resulting in multi-layering of ductal epithelium (b and c); in ductal carcinoma *in situ*, there are still tumour cells remaining connected by intercellular junctions between neighbouring ones, and this type of BC consists of malignant cell masses that lack ductal organization, but remain restricted to breast ducts, and are surrounded by both myoepithelial cells and an intact BM (d); finally, invasive ductal carcinoma is characterized by an extensive growth of the malignant cells beyond the ductal structure and the BM, complete loss of cell-cell adhesion between cancer cells, with the exception of the tumour cells that invade the surrounding tissue as clusters, reduced number of myoepithelial cells, and breaching of BM, which consequently lead to infiltration of the breast ducts by tumour cells and grow into the surrounding tissue (e).

epithelial cadherin (E-cadherin), and the cytosolic catenins. Basally to AJs are desmosomes that provide mechanical stability by anchoring cytoskeleton keratinous intermediate filaments to the plasma membrane and facilitate cell-to-cell communication

through signal transmission (Brooke et al. 2012, Owens et al. 2013). The proteins of this type of intercellular junctions include transmembrane adhesion proteins (desmoglein and desmocollin) that belong to the cadherin family, and cytosolic proteins (desmoplakin, plakoglobin, and plakophilin) that form the desmosomal plaque. This cytoplasmic plaque is responsible for connecting the cytoskeleton to the transmembrane adhesion proteins (Brooke et al. 2012). Desmosomes are also responsible for the attachment of epithelial cells to myoepithelial cells (Gudjonsson et al. 2005). Finally, less regularly organized, GJs are unique intercellular channels that allow diffusion of small molecules, being essential for the communication between neighbouring cells (Giepmans and van Ijzendoorn 2009). These channels are composed by the transmembrane proteins connexins (Cx), wherein the Cx43 is the most abundantly expressed in humans. Similarly to other intercellular junctions, GJs also establish interactions with the cytoskeleton, namely through microtubules and actin, which increase GJ stability (Giepmans 2006, Giepmans and van Ijzendoorn 2009). Regarding cell-BM interactions, they are established by junctional complexes like hemidesmosomes, which anchor the cells to the underlying BM, and maintain tissue polarity (Borradori and Sonnenberg 1999). The hemidesmosomes are composed by three classes of proteins: the cytoplasmic plaque proteins (bullous pemphigoid antigen 1 isoform e and plectin) responsible for the linkage of cytoskeleton to the cell surface; the transmembrane proteins [integrin α6β4, bullous pemphigoid antigen 2, and cluster of differentiation (CD)151] that act as cell receptors connecting the cell and the ECM, and the BM-associated proteins (laminins, such as laminin-332) of the ECM (Borradori and Sonnenberg 1999, Walko et al. 2015). In mammary duct, hemidesmosomes bind myoepithelial cells to BM (Gudjonsson et al. 2005). Since the myoepithelial cells form a discontinuous layer, breast epithelial cells can also interact with the BM through hemidesmosomes and proteoglycan adhesion complexes, whose proteins are coupled to the cytoskeleton and associated to signalling pathways that control cell fate (Bergstraesser et al. 1995, Muschler and Streuli 2010). Thus, the regulation of cell-cell junction complexes and epithelium polarity ensures structural and functional differentiation of the epithelial mammary tissue and their deregulation is associated with cancer. In fact, alterations in desmosomes, GJs, and hemidesmosomes have been increasingly associated with BC progression (Bergstraesser et al. 1995, Brooke et al. 2012, Owens et al. 2013), whereas TJs and AJs are involved in the migration of malignant cells across the endothelium (Martin et al. 2002, Arvanitis et al. 2014).

It is consensual that the development of a primary breast tumour is a multistep process, although no consensus has still been reached on the steps and features of each stage (Ellis 2010, Bombonati and Sgroi 2011). For the ductal type BC progression, the classical ductal model proposes that evolution initiates in normal epithelium, progresses from benign flat epithelial atypia to atypical ductal hyperplasia, evolves into malignant ductal carcinoma *in situ*, and finally to invasive ductal carcinoma (Ellis 2010, Bombonati and Sgroi 2011), as schematically depicted in Figure 1. In addition to the mentioned phases, an alternative model proposes an intermediate step before the progression to flat epithelial atypia, a phase called usual ductal hyperplasia (Bombonati and Sgroi 2011). However, immunohistochemical and molecular biological evidences strongly support that this alternative model of ductal BC progression is likely invalid (Ellis 2010, Bombonati and Sgroi 2011). Thus, according to classic ductal model, both epithelial atypia and hyperplasia are precursor lesions characterized by intraductal proliferation of epithelial cells, resulting in multi-layering of ductal epithelium. Ductal carcinoma *in situ* is the most common type of non-invasive BC. This type of BC is another intraductal proliferative lesion characterized by lacking ductal organization, but remains restricted to breast ducts without invasion through the BM into the surrounding breast stromal compartment (Ellis 2010, Pinder 2010, Bane 2013). The tumour cells that compose the primary tumour still express E-cadherin that is progressively lost with disease progression, and are surrounded by both a layer of myoepithelial cells and an intact BM. Lastly, the invasive ductal carcinoma consists in an extensive growth of the malignant cells beyond the ductal structure and the BM (Sgroi 2010). Although still under debate, increasing evidence suggests that the progression of carcinoma *in situ* to the invasive type is related to activation of the EMT that allows malignant cells to migrate and escape from the primary tumour (Knudsen et al. 2012). So, invasive ductal carcinoma is characterized by complete loss of cell-cell adhesion, reduction in myoepithelial cell number, and breaching of BM, which consequently lead to infiltration of the breast ducts by tumour cells and migration into the surrounding tissue (Gusterson et al. 1982, Debnath et al. 2003, Kominsky et al. 2003). Therefore, invasive cells have the potential to spread into lymphatic and/or blood vessels and finally form metastases in other organs.

### 2.1. Epithelial-mesenchymal transition

To date, the EMT has been the phenomenon that better explains distant metastases formation by epithelial cancers, such as BC. EMT is a reversible process whereby an

epithelial cell acquires mesenchymal features (Yang and Weinberg 2008), as depicted in Figure 2. In contrast to epithelial cells, mesenchymal cells usually are not involved in cell-cell interactions and lack the apical-basal polarity (Greenburg and Hay 1982). Thanks to these features, mesenchymal cells have a higher invasiveness than epithelial cells. Initially, carcinoma cells lose the junctions that join them to other cells and/or to BM. Hence, BCCs downregulate the expression of epithelial marks, such as the transmembranar E-cadherin and the intermediate filament pan cytokeratin, followed by enhanced production of mesenchymal proteins, including neuronal cadherin (N-cadherin) and vimentin (transmembrane adhesion molecule and intermediate filament protein, respectively) (Onder et al. 2008, Lv et al. 2013). The switch between E-cadherin and N-cadherin induces the resistance to anoikis, a programmed cell death induced by loss of cell adhesion, through the modulation of pro- and anti-apoptotic genes (Onder et al. 2008, Paoli et al. 2013). Thus, the ability to overcome anoikis is correlated with the acquisition of the mesenchymal phenotype, allowing the survival and proliferation of cancer cells without cell-cell interactions. In addition, activation of EMT is accompanied by a breakdown of the BM due to increase of protease secretion, such as MMPs, and alterations in the production of BM proteins (Tester et al. 2001, Ota et al. 2009, Espinosa

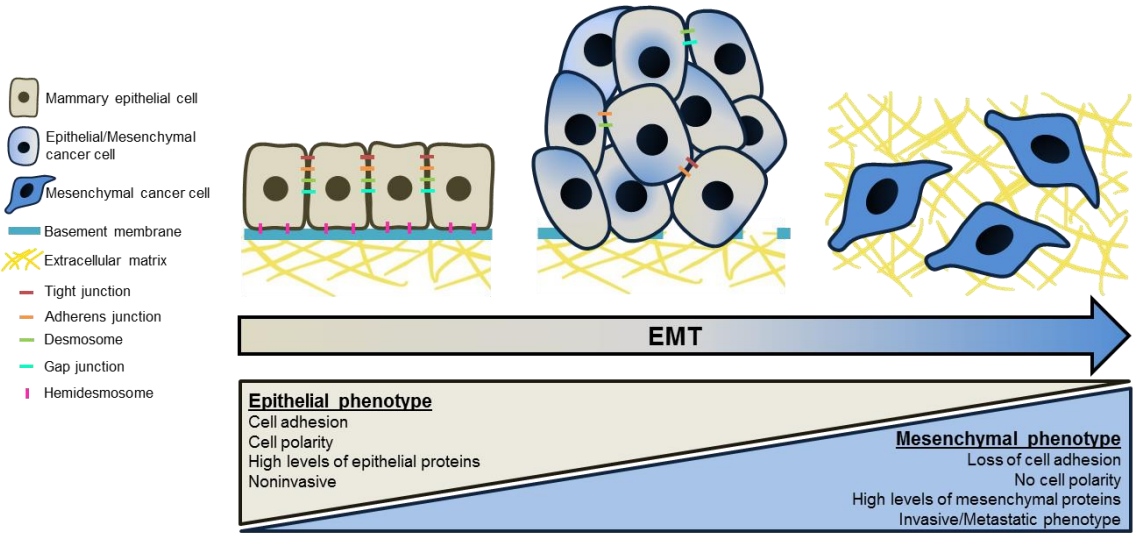


**Fig. 2** Schematic representation of epithelial-mesenchymal transition (EMT), a process by which epithelial breast cancer cells acquire mesenchymal characteristics. Mammary epithelial cells are connected to each other through intercellular junctions (tight junctions, adherens junctions, desmosomes, and gap junctions), as well as to the basement membrane (BM) by hemidesmosomes. During EMT, mammary epithelial cells lose cell adhesion and polarity, and consequently stability, acquiring invasive ability. Along this process, the expression of epithelial proteins is decreased, whereas that of mesenchymal proteins is increased, the morphology changes from cuboidal/cylindrical to fusiform. In parallel with the changes in the morphogenesis and architecture of the mammary ducts, the BM undergoes disruptive changes.

Neira and Salazar 2012). These alterations lead to a loss of polarity, a reorganization of cytoskeletal constituents, and an acquisition of mesenchymal-like phenotype, essential for their entry into blood or lymphatic stream.

Although EMT allows malignant cells to change their shape and motility, not all BCCs complete this process, retaining certain epithelial characteristics. This fact is supported by the co-expression of mesenchymal and epithelial markers in BCCs, allowing them to have simultaneously adhesion and migratory properties (Yu et al. 2013). In addition, oestrogen binding to ER also promotes intercellular adhesion via upregulation of the desmosomal proteins and enhancement of formation of desmosomes (Maynadier et al. 2012), increasing the cell-cell attachments that allow tumour cells to move and invade collectively. Thus, some malignant cells can form multicellular clusters even in primary tumour mass. Collective migration of grouped cells that maintain their cell-cell interactions has been implicated in cancer metastasis, and may favour the survival of BCC clusters in circulation. Once in the circulatory system, cancer cells are called circulating tumour cells (CTCs) and can be detected as single cells and as multicellular clusters termed as tumour emboli (Bidard et al. 2010, Tsoi et al. 2010). Both individual and clusters of CTCs can interact with platelets, which protect the malignant cells against the immune response (Gay and Felding-Habermann 2011, Roop et al. 2013). Such interaction with platelets also enhances chemotaxis and chemoinvasion, and posteriorly facilitates extravasation. However, since CTCs are probably targets of a selective process responsible for eliminating carcinoma cells with a lower metastatic potential, the tumour embolus presents a higher ability to form new tumours compared to an individual cell (Aceto et al. 2014). Due to their higher dimensions and resistance to apoptosis compared to an individual cell, multicellular clusters are more likely not only to survive during the way up to the organ that these cells will metastasize, but also to be trapped in small capillaries of secondary sites.

Regardless of the type of EMT, many genes are transcriptionally altered in order to coordinate the repression of epithelial proteins. This repression is orchestrated by regulators such as the transcription factors Twist, Snail (zinc finger protein snail 1), Slug, and ZEB (zinc finger E-box-binding homeobox) family (Samatov et al. 2013). Among the EMT transcription factors, Twist has the ability to promote the formation of invadopodia, specialized actin-based membrane protrusions found in malignant cells that facilitate the local invasion through focal ECM degradation (Eckert et al. 2011). In addition, the regulators of EMT mediate several signal transduction pathways, including

transforming growth factor beta (TGF-β) and Notch1 signalling, which in turn promote metastasization.

So, during the transition from *in situ* to invasive BC, the primary tumour cells exhibit mesenchymal characteristics that allow them to survive without cell-cell interactions and to degrade the BM, resulting in migration and local invasion of mammary tissue.

### 2.2. Local invasion

Invasion is one of the main processes of metastatic cascade, being not only a fundamental step in tumour progression, but also a driving force for metastasis. In ductal carcinoma *in situ*, the cancer cells reside within a primary tumour site well-confined by myoepithelial cells and a BM that oppose tumour growth and invasion (Sternlicht et al. 1997, Hu et al. 2008). However, when the tumour progresses to an invasive form, the number of myoepithelial cells reduces at sites of invasion, becoming the minor component of invasive ductal carcinomas (Gusterson et al. 1982). In addition, malignant cells undergoing EMT induce the secretion of proteases that are responsible for degrading the BM. This component of mammary gland plays an important role in invasion of BCCs, since the BM is a reservoir of growth factors that are released during its degradation by tumour cell-secreted proteases (Vukicevic et al. 1992, Hu et al. 2008). Moreover, the BM also plays pivotal roles due to its involvement in pathways initiated by integrin-mediated cell-matrix adhesions that induce intracellular signals and lead to alterations in cell polarity, survival, proliferation, and migration (Nisticò et al. 2014). In addition to myoepithelial cells and BM, even normal mammary epithelial cells promote the invasion of neighbouring tumour cells by secreting soluble laminin, a BM protein, as recently suggested (Lee et al. 2015). The continuous production of laminin induces long membrane cellular protrusions in BCCs, which in turn allow the contraction and subsequently invasion of the surrounding matrix by malignant cells. So, although the precisely controlled tissue architecture of normal epithelium serves as an intrinsic barrier to invasiveness, this may be not enough to stop the cascade that will culminate in metastasization of a secondary site.

Tumour cells can have a diversity of invasive strategies, which are closely related to EMT. When a cancer cell undergoes a complete EMT and acquires the mesenchymal phenotype, this cell invades singly into the surrounding tissue. However, as mentioned above, some malignant cells maintain epithelial characteristics, preserving cell-cell

junctions, and this strategy allows the BCC cluster to invade and migrate collectively. These two different strategies of invasion can also coexist within the same tumour (Friedl et al. 1995). Curiously, cancer cells can also undergo transition between invasion modes through the switch between activation and blockade of TGF-β signalling associated to single cell movement and collective cell invasion, respectively (Giampieri et al. 2009). Regardless of the chosen strategy of invasion, the proteolytic destruction of the BM is essential for migration and invasion of tumour cells. Once motile BCCs have dissolved the BM, they enter in mammary stroma where they interact with stromal cells that facilitate the tumour progression.

In normal morphogenesis, mammary ducts are surrounded by the stroma formed by the ECM and a wide variety of cell types, including adipocytes, fibroblasts, and macrophages (Sternlicht et al. 2006). The ECM is the major component of mammary gland microenvironment and, similarly to the BM, regulates epithelial architecture and function (Muschler and Streuli 2010). The cancer progression is dependent on the remodelling and stiffening of the ECM surrounding the tumour cells (Levental et al. 2009, Ko et al. 2016). The remodelling of this matrix is associated to a crosslinking of collagen (the most abundant ECM scaffolding protein), which in turn stiffens the ECM, promotes focal adhesion, and induces invasion and tumour progression (Levental et al. 2009). In addition, aggressive malignant cells are able to alter the mechanical environment of the matrix through the secretion of bioactive lipids that increase ECM stiffness (Ko et al. 2016). Moreover, carcinoma cells release MMPs, such as MMP-2 and MMP-9, that degrade the ECM and augment the bioavailability of ECM-derived growth factors (Tang et al. 2005). One of these growth factors is VEGF that attracts ECs to migrate into areas of active tumour cell proliferation, where cancer cell-induced cleavage of ECM components results in increased vascularization (Lee et al. 1998, Tang et al. 2005). In addition, the degradation of ECM components by invasive tumour cells can lead to an extensive endothelial anoikis, and to attraction and migration of metastatic cells through apoptotic endothelium (Peyri et al. 2009). Thus, deregulation of ECM stiffness and dynamics enhances cancer cell survival, proliferation, migration, and invasion, playing an essential role in BC progression. Besides ECM, stromal cells also have an important role in invasion. Regarding adipocytes, the crosstalk and interaction between these stromal cells and malignant cells lead to an activated phenotype in adipocytes, named cancer-associated adipocytes (Dirat et al. 2011). In turn, this activation results in overexpression of proteases and proinflammatory cytokines by adipocytes that stimulate

the invasiveness of tumour cells. Curiously, adipose cells can also promote the resistance of BCCs to cellular cytotoxicity by therapies such as trastuzumab (Duong et al. 2015), thus ensuring that the malignant cells continue to invade the breast tissue. Local invasion leads to the proximity of cancer cells and adipocytes, which respond to this proximity by phenotypical changes that generate fibroblast-like cells (Bochet et al. 2013). The fibroblasts are stromal cells whose functions are maintaining the mammary ECM intact through the synthesis of ECM components, such as collagens, proteoglycans and fibronectin, and regulating the differentiation and homeostasis of adjacent epithelia (Kalluri and Zeisberg 2006, Inman et al. 2015). In tumour stroma, BCCs recruit and activate the majority of fibroblasts, termed as cancer-associated fibroblasts, which result in ECM remodelling to create tracks for tumour cell migration (Gaggioli et al. 2007, Avgustinova et al. 2016). The generation of cancer-associated fibroblasts depends on TGF-β signalling in response to Wtn7a, a factor secreted by aggressive malignant cells that drives the acquisition of a desmoplastic response (Avgustinova et al. 2016). Host macrophages are phagocytic cells that have an important role in host defence against pathogens (Paape et al. 2000) and, like the other stromal cells, also participate in local invasion. This initial step of BC progression depends on both paracrine signalling with macrophages as well as autocrine signalling involving the tumour cells themselves (Patsialou et al. 2009). On one hand, macrophages secrete epidermal growth factor (EGF), which promotes the formation of elongated protrusions that guide malignant cells toward blood vessels, this way favouring the invasion process (Goswami et al. 2005, Wyckoff et al. 2007). On the other hand, tumour cells secrete the cytokine colony-stimulating factor, and sense and stimulate EGF secretion by macrophages. Thus, all stromal components contribute, directly or indirectly, to the movement of BCCs toward lymph nodes and blood vessels, as will be addressed below.

Dynamic interaction between cancer cells and the surrounding microenvironment of mammary ducts is essential to potentiate the malignant behaviour of carcinoma cells. Since the influence exerted by the stromal components remains largely unknown, detailed characterization of these components will provide further evidence of their critical roles in BC progression. However, it is unquestionable that the interactions between cancer cells and the surrounding microenvironment are essential for their entry into the stroma of mammary gland and move directly towards the systemic circulation. Once near a blood or lymphatic vessel, intravasation of tumour cells can occur and, if they survive in circulation, dissemination to distant sites may take place.

### 2.3. Intravasation

As mentioned above, malignant cells detach from the primary tumour mass by undergoing EMT and then invade the surrounding microenvironment of mammary duct; once in the stroma of the mammary gland, the stromal components, such as fibroblasts and macrophages, guide the cancer cells towards vessel wall. The subsequent entry of cancer cells into the circulatory system is called intravasation (Wyckoff et al. 2000). Cancer cells can escape from the primary tumour via two main routes of dissemination: haematogenous or lymphatic (Markiewicz et al. 2013). The decision to intravasate into either blood or lymphatic vessels may depend on the following factors: anatomical differences of the blood and lymphatic vessels; invasion strategy utilized by tumour cells (individual movement versus collective migration); movement promoted by stromal cells; active mechanisms that attract cells to a specific type of vasculature; induction of angiogenesis and/or lymphangiogenesis; and the effect of fluid shear stress on cell survival in circulation (Wong and Hynes 2006, Bockhorn et al. 2007).

Although both blood and lymphatic vessel walls are lined by ECs, there are anatomical differences between them that may influence the intravasation of the malignant cell. Similarly to epithelial cells, the ECs of blood vessels are also connected by cell-cell junctions although lacking desmosomes (Wallez and Huber 2008). These ECs are embraced by some pericytes, perivascular cells that wrap around blood capillaries and reinforce vascular structure, and both ECs and pericytes are embedded within the BM (Fujiwara and Uehara 1984, Davis and Senger 2005). Thence, the anatomical barriers of blood vessels appear to be more hardly penetrated by individual cancer cells. Thus, when the intravasation occurs through the blood vessels, invasive cells must induce cellular and molecular changes to promote their cross through BM, pericyte, and EC barriers. In contrast, ECs that compose the lymphatic capillaries lack tight intercellular junctions, are not embraced by pericytes, and are surrounded by a discontinuous BM (Tanis et al. 2001). Indeed, the higher permeability of lymphatic vessels may favour the passage of individual and mainly clusters of tumour cells (Byers et al. 1995). Despite the migration of cancer cell clusters through blood vessels has not been described, the observation of tumour emboli in bloodstream of BC patients (Tsoi et al. 2010) raises two hypotheses: whether cancer cells intravasate under the form of clusters or, once in bloodstream, aggregate with components and cells present in blood circulation. In addition, stromal cells of surrounding microenvironment can promote a preferential movement of carcinoma cells to blood vessels, since the interaction between cancer cell and stromal ones to promote

lymphatic intravasation has never been reported. Like local invasion, the haematogenous intravasation is facilitated by macrophages. Macrophages occupy a perivascular localization in vessel surface, and tumour cells are attracted by the macrophage-derived EGF (Wyckoff et al. 2007). This attraction probably explains why malignant cells are more likely to migrate toward blood vessels than lymphatic vessels in mammary tumours. In addition, macrophage-derived VEGF-A, a growth factor that induces EC proliferation and promotes cell migration, causes local disruption of EC junctions, which hence leads to transient vascular permeability of the blood vessel (Harney et al. 2015). Thus, the interaction between perivascular macrophages and BCCs forms chemoattractive gradients that augment the recruitment of malignant cells towards the blood vessel wall, and facilitate the transendothelial crossing through vascular endothelium. The availability of blood and/or lymphatic vessels in the surrounding area of tumour cells may also influence the pathway taken for dissemination. Both angiogenesis and lymphangiogenesis are induced by the same growth factors, such as VEGFs (Alitalo et al. 2005, Shibuya 2006). Hence, tumours might concomitantly induce angiogenesis and lymphangiogenesis; however, lymphangiogenesis has been less documented in BC compared to angiogenesis (Wong and Hynes 2006). Finally, the fluid shear stress plays a pivotal role in cell survival within the circulation and differs between bloodstream and lymphatic stream. Whereas tumour cell survival may benefit from the low-shear system of fluid transport characteristic of lymphatic vessels, the increased haemodynamic flow rate may also help individual cells to reach distant organs (Byers et al. 1995). Thus, the dissemination via the haematogenous circulation appears to represent the major mechanism by which invasive BCCs are able to spread and form secondary tumours.

The intravasation through blood vessels occurs in the abluminal to luminal direction and involves important interactions between the invasive cancer cell and the EC that composes vessel walls, as schematically depicted in Figure 3. Although the intravasation is considered one of the key steps of the metastatic cascade, this interaction between tumour cells and ECs remains poorly characterized, not only because the transendothelial migration (TEM) is a localized and transient process, but also because the *in vitro* models used to study this process are only useful to understand and identify molecular components and signalling pathways involved in intravasation (Khuon et al. 2010). Prior to TEM, adhesion of cancer cell to EC must occur to approach these two cellular types and facilitate the translocation of the tumour cell into the circulation.

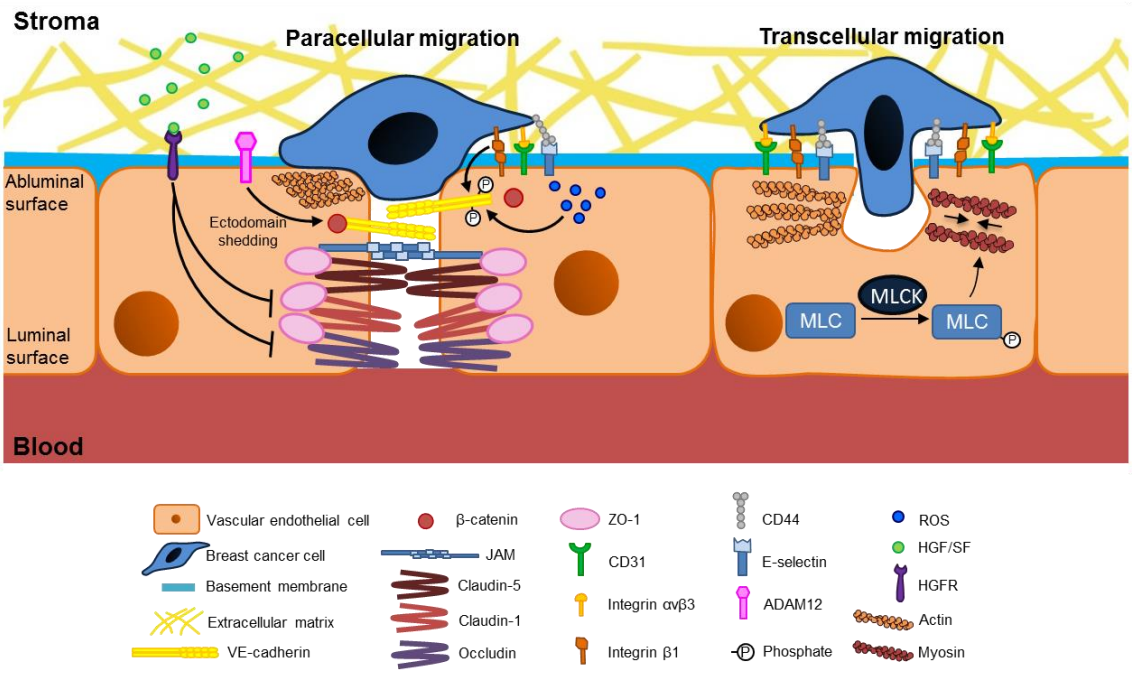


**Fig. 3** Schematic representation of the transendothelial migration (TEM) pathways during the intravasation process. Breast cancer cell (BCC) can cross endothelial cells (ECs) to enter into the blood circulation by paracellular or transcellular routes. This is a multistep process that involves interactions between BCC and ECs through the binding of specific ligands to the corresponding receptors. Vascular ECs express membrane receptors such as cluster of differentiation (CD)31, endothelial selectin (E-selectin), and integrin β1 that interact with the respective ligand integrin αvβ3, CD44, and integrin β1 expressed by BCC. The adhesion step approaches the cancer cell to the vascular endothelium and facilitates the TEM. During paracellular migration, there is a disruption and reorganization of intercellular junctions and also a reorganization of EC cortical actin cytoskeleton around the invasion site. The ectodomain shedding, a mechanism that compromises the integrity of cell-cell junctions, of the adherens junction protein vascular endothelial (VE)-cadherin is promoted by a disintegrin and metalloproteinase (ADAM)12, and the phosphorylation of this protein is induced by both adhesion step via integrin β1 and reactive oxygen species (ROS). In addition, adhesion via integrin β1 promotes the dissociation of β-catenin from the VE-cadherin/catenin complex. Regarding tight junctions (TJs), the hepatocyte growth factor/scatter factor (HGF/SF), a cytokine secreted by stromal cells, interacts with its receptor, HGFR, expressed in ECs surface. The interaction between HGF/SF and HGFR reduces the expression of the TJ proteins claudin-1, occludin, and zonula occludens (ZO)-1, but not of junctional adhesion molecules (JAMs) and claudin-5. On the other hand, during transcellular migration, the border of the EC remains intact since the migration of BCC occurs when the EC envelopes the malignant one. Firstly, there is a reorganization of the EC actin filaments that circumscribe the invasion pore. Then, EC myosin organizes into a ring-like invasion array via activation of endothelial myosin light chain kinase (MLCK). This enzyme phosphorylates its myosin light chain (MLC), leading to myosin contraction. Thus, the rapid cytoskeletal and membrane remodelling creates a transitory pore-like structure that encapsulates the BCC and facilitates its migration across the EC.

Curiously, the cell surface adhesion receptors expressed by vascular and lymphatic ECs

are different (Dua et al. 2005), limiting the process of intravasation and consequently haematogenous or lymphatic dissemination only to cancer cells that express the respective ligands. Among the membrane receptors expressed by vascular ECs are the CD31 (also known as platelet endothelial cell adhesion molecule-1, PECAM-1), endothelial selectin (E-selectin), and integrin β1, which interact respectively with integrin αvβ3, CD44, and integrin β1 expressed by BCCs (Price et al. 1996, Bauer et al. 2007, Zen et al. 2008, Romagnoli et al. 2014). Once the adhesion interactions are established between both endothelial and cancer cells, the BCC is able to migrate across the vascular endothelium. So far, TEM of BCCs was reported to occur by two types of pathways, both supported by ECs: paracellular, i.e. passage through the intercellular junctions of two ECs, and transcellular, a transport through the body of an EC that is also called transcytosis (Khuon et al. 2010, Arvanitis et al. 2014). Thus, the paracellular migration requires the interaction of cancer cells with endothelial junctions to cross the endothelium. Firstly, cancer cells interact with AJs, due to their basal localization, and then with TJs. During paracellular migration, there is a clear disruption of the AJs protein vascular endothelial (VE)-cadherin and reorganization of EC cortical actin cytoskeleton around the invasion site to facilitate the transmigration (Arvanitis et al. 2014). The ectodomain shedding of VE-cadherin is one mechanism that rapidly releases membrane-anchored proteins from the cell surface and converts into soluble proteins (Hayashida et al. 2010, Fröhlich et al. 2013), leading to disruption of the AJs. This mechanism is mediated by a cell-surface protease that is expressed in vascular ECs, a disintegrin and metalloproteinase (ADAM) 12. In addition, both tyrosine phosphorylation of VE-cadherin and dissociation of β-catenin from the VE-cadherin/catenin complex are triggered by adhesion via integrin β1 (Haidari et al. 2012). On the other hand, the generation of reactive oxygen species resulting from the interaction of BCC with vascular ECs can also induce phosphorylation of VE-cadherin (Haidari et al. 2013). Regarding TJs, although there are no apparently changes in distribution or expression of both junctional adhesion molecules and claudin-5, the expression of claudin-1, occludin, and ZO-1 is reduced, leading to a decreased transendothelial resistance and an increased paracellular permeability (Martin et al. 2002). This increased paracellular permeability is induced by the hepatocyte growth factor/scatter factor, a cytokine secreted by stromal cells that is involved in cancer cell migration and invasion. The hepatocyte growth factor/scatter factor requires activation by hepatocyte growth factor activator expressed in large amounts by invasive BCCs, and then interacts with the hepatocyte growth factor

receptor (also known as c-Met) expressed in ECs surface (Martin et al. 2002, Sierra and Tsao 2011). Besides the migration between two adjacent vascular ECs, BCCs were reported to cross the endothelial barrier through individual ECs (Khuon et al. 2010). In contrast to paracellular pathway, during transcellular migration, the border of the EC remains intact since the migration of cancer cell starts to occur when the EC begins to envelope the invasive cell (Arvanitis et al. 2014). As transcellular TEM progresses, the malignant cell migration requires drastic reorganization of EC actomyosin system, a complex composed by both actin and myosin filaments that plays an important role in cell contractility (Khuon et al. 2010, Arvanitis et al. 2014). Firstly, at invasion site, there is a local reorganization of the EC stress fibres, the major contractile structures constituted by actin filaments denuded of myosin, to circumscribe the invasion pore. Some of these stress fibres eventually undergo stress-induced fracture and recoil to form a much larger pore (Arvanitis et al. 2014). Secondly, EC myosin organizes into a ring-like invasion array via localized activation of endothelial myosin light chain kinase (Khuon et al. 2010, Arvanitis et al. 2014). This enzyme phosphorylates its myosin light chain, leading to myosin contraction. Subsequently, the rapid cytoskeletal and membrane remodelling creates a transitory pore-like structure that encapsulates the invading cancer cell and facilitates its migration across the EC (Khuon et al. 2010). As the tumour cell passes the invasion site, the EC cytoskeleton progressively returns to its initial organization (Arvanitis et al. 2014). Thus, intravasation of malignant cells within circulation system is a host-tumour interaction, where the endothelium plays a pivotal role in tumour invasive potential.

So, after the BCCs acquire the ability to infiltrate the mammary duct and invade the surrounding microenvironment, these cells have the opportunity to enter into the circulatory system. Cancer cell intravasation can be considered a rate-limiting step of the metastatic process, regulating the number of malignant cells that cross the vascular endothelium. Once within the circulatory system, invasive cancer cells migrate out of the primary tumour site until reaching the vasculature of the secondary organs where they will form new tumours. In order to spread, malignant cells must survive during their journey within the bloodstream.

### 2.4. Survival in circulation

The microenvironment within the bloodstream and the wide distance between the mammary gland and a distant secondary organ are hostile for CTCs, turning their survival

into a great challenge. Firstly, the fluid shear stress and increased haemodynamic flow rate of the bloodstream, as well as the presence of immune cells especially natural killer cells, can result in death of a great proportion of tumour cells. Conveniently, carcinoma cells have the ability to escape from immune cell-induced death through cooperative host-tumour interactions (Labelle and Hynes 2012). Regarding natural killer cells, BCCs are able to induce the reduction of function of these immune cells (Mamessier et al. 2011). The decreased function of natural killer cells is associated with a decreased expression of their activating receptors and an increased expression of inhibitory receptors. In addition, tumour cells can benefit from the rapid coating provided by platelets, forming a cluster that protects the BCCs from cytotoxicity mediated by natural killer cell antitumour reactivity (Kopp et al. 2009). The interaction between platelets and carcinoma cells is induced by the expression of cell adhesion molecules in platelets, which bind to cell surface molecules expressed by malignant cells (Aigner et al. 1997, Kopp et al. 2009). Thus, platelet-coated cancer cells are more likely to persist within the circulation until they arrest at distant tissue vasculature, an event whose occurrence may be further increased due to the large diameter of the tumour cells.

The amount of time that malignant cells spend in circulation is believed to be short (Aceto et al. 2014), not only because these cells are subjected to shear stress and immune cells that eliminate carcinoma cells with a lower metastatic potential, but also because CTCs, especially clusters, are rapidly entrapped within capillaries of distant organs. Indeed, given the relatively large diameters of BCCs (12–17 μm) (Yusa et al. 2014) and the diameter of a capillary (3–30 μm) (Sarveswaran et al. 2016), it is likely that a vast majority of tumour cells become trapped in smaller capillaries. In addition, the size of the individual cells and clusters of cells is inversely proportional to velocity of cells in bloodstream (Au et al. 2016). Thus, aggregates of cancer cells transit in blood vessels at low velocity compared to single cells, which increases the probability of their entrapment in vessels even with larger diameters. Therefore, it is crucial to understand all the mechanisms behind the formation of these clusters to prevent the aggregation of malignant cells and/or their entrance into the ‘target organ’.

After intravasation, survival of cancer cells into the circulation is a crucial step for tumour cells, since most of them fail to remain viable. So, only malignant cells with high metastatic potential survive and succeed in extravasation and dissemination into other organs, such as the brain.

## 3. Brain metastasization

Breast to brain metastasis follows a vascular distribution mainly through haematogenous spread (Lin et al. 2004). The distribution of brain metastasis depends on blood flow and tissue volume, being detected 52-62% in the cortex, 30% in central brain (which includes hypothalamus, thalamus, midbrain, caudate putamen, ventral striatum, and basal forebrain), 5-13% in posterior brain (which includes cerebellum, pons, and medulla), and 4% in olfactory regions (Perera et al. 2012). Commonly, BC patients with brain metastases exhibit multiple tumours, although they can also present solitary and/or leptomeningeal metastases (Evans et al. 2004). The formation of these secondary tumours can lead to an impairment of neurocognitive functions related to executive function, motor dexterity, and learning/memory (Chang et al. 2007), which may be a consequence of interactions between cancer cells and host cells that compose the brain microenvironment and the occupation of brain areas responsible for those functions by well-established metastases that can reach 4 cm of diameter.

The arrival of viable malignant cells to the brain can result simply from a physical trapping of CTCs in small blood vessels of this secondary organ or from a trapping facilitated by migration of tumour cells driven by organ-derived chemoattractants (Hujanen and Terranova 1985, Kienast et al. 2010). Chemokines and their receptors play a key role in organ-specific metastasis development and one of the most studied pairs is the cysteine-X amino acid-cysteine receptor 4 (CXCR4) and its ligand stromal cell-derived factor-1α (also known as CXCL12) (Müller et al. 2001, Lee et al. 2004, Salmaggi et al. 2014). The chemokine CXCL12 is synthetized by brain microvascular ECs (BMVECs) to attract CXCR4-positive cells, such as BCCs (Müller et al. 2001, Liu et al. 2014). In addition, CXCL12 increases vascular permeability, leading to blood vessel instability and consequently allowing the TEM through brain endothelium (Lee et al. 2004). Thus, CXCL12/CXCR4 axis plays an important role in the homing of carcinoma cells from primary to secondary site, contributing to metastatic progression.

Once reaching the ‘target organ’, CTCs arrest within the brain vasculature in small capillaries, especially at vascular branch points, where the slower flow allows the interactions with BMVECs and posterior extravasation (Kienast et al. 2010, Stoletov et al. 2010). Both arrest and extravasation of cancer cells are followed by early, immediate, and drastic changes in the brain microenvironment, which promote the growth of metastatic tumour cells and their progression to well-established metastasis (Lorger and

Felding-Habermann 2010). Since the secondary organ is the brain, the first host cell type that extrasavating cancer cells encounter is the BMVEC that composes the BBB (Wilhelm et al. 2014). Although this highly selective barrier limits the penetration of both solutes and cells due to its elaborate intercellular junctions together with low pinocytic vesicles, the evidence that some metastatic cells can cross the BBB is the formation of metastases in the brain. The BCCs preferentially metastasize to this secondary organ probably due to the fact that, once inside the brain, the BBB not only protects them from the immune surveillance of the organism and from chemotherapeutic drugs, but also produces substances favourable for their survival and subsequent proliferation. Accordingly, the development of brain metastasis has been also correlated with the aggressiveness of BCCs (Yonemori et al. 2010). Whereas triple-negative cancer cells are more aggressive and have the ability to induce the disruption of the BBB, the HER2-positive cells may develop brain metastasis based on their potential to migrate through ECs, maintaining the BBB properties. The integrity of BBB is also directly associated to the size of brain lesions (Zhang et al. 1992). In fact, in an *in vivo* brain metastasis model, when BCCs form lesions smaller than 0.1 $mm^2$, the BBB remains intact; however, when metastatic tumour mass exceeds 0.5 $mm^2$, the large size of these metastases also compromised structurally and functionally the BBB. In addition, the increased permeability of BBB can result from the disturbance of interactions established between BMVECs and astrocytes caused by increasing growth of the tumour mass (Zhang et al. 1992). Thus, although tumour cells can induce the BBB disruption, the integrity of this barrier might remain intact or even be repaired after the passage of metastatic cancer cells into the brain.

As mentioned above, the arrest of cancer cells in brain capillaries precedes the extravasation of these cells into the 'target organ'. The migration of malignant cells across the brain endothelium is a process highly dependent on interactions established between tumour cells and the BBB endothelium. When the extravasation of carcinoma cells occurs successfully, these cells can proliferate and colonize the brain giving rise to the formation of new tumours.

### 3.1. Extravasation and role of the blood-brain barrier

Intravasation and extravasation are two similar processes, since both require the TEM of BCCs across the EC barrier. However, the approach to the endothelium by malignant cells is from opposite sides of vasculature. The extravasation of BCCs is the passage of highly metastatic CTCs from the bloodstream into the brain through the BBB.

Localized between peripheral circulatory system and the neural tissue, the BBB controls the cerebral homeostasis, and protects the CNS against neurotoxicity and entry of pathogens (Ghersi-Egea et al. 1995). This barrier is composed by BMVECs that establish interactions with each other through elaborate intercellular junctions that restrict the paracellular permeability (Cardoso et al. 2010). However, the formation of brain metastasis suggests that the barrier properties of BMVECs are not enough to prevent the cross of certain BCCs through the BBB, and hence allow their entrance into the brain.

There are three steps involved in extravasation of cancer cells into the brain: rolling, firm adhesion and, finally, paracellular TEM, as schematically depicted in Figure 4. Although the duration of *in vitro* TEM is estimated to be 6 to 18 hours, the entire extravasation process requires 3 to 5 days to be completed (Lorger and Felding-Habermann 2010). For the occurrence of extravasation, cancer cells must slow down, arrest in capillaries with a similar diameter to that presented by the cells, and roll along BMVECs surface, establishing several receptor-ligand interactions with ECs (Kienast et al. 2010, Stoletov et al. 2010). Although CTCs are subjected to shear forces resulting from blood flow, they can migrate on the blood vessel wall with or against the flow due to the generation of string traction forces and membrane protrusions (Stoletov et al. 2010, Spencer and Baker 2016). Thus, during the rolling, malignant cells search for the best site for crossing the BBB, and establish transient interactions with BMVECs. Curiously, when cancer cells arrest in BMVECs they present an elongated shape that later rounds up, enabling the metastatic cells to stretch across the vessel wall (Lorger and Felding-Habermann 2010). Similarly to leukocyte recruitment to the endothelium, the rolling of CTCs is mediated by selectins, in particular by E-selectin and their ligands (Soto et al. 2014, Mondal et al. 2016). Selectins are not normally expressed on quiescent BMVECs, but, when stimulated by inflammatory cytokines secreted by cancer cells or cancer cell-associated leukocytes, the expression of these adhesion molecules augments in ECs surface (Wong and Dorovini-Zis 1996, Kang et al. 2015). At the same time, tumour cells express glycoprotein ligands for E-selectin, including platelet selectin glycoprotein ligand-1, CD24, and tetrasaccharide sialyl Lewis x (Myung et al. 2011, Soto et al. 2014). Once the first interaction between BCCs and ECs is established, firm and stable adhesion between these two cellular types can take place. E-selectin also plays an important role in this step of extravasation, while binding to other ligands expressed by tumour cells, such as CD44 and mucin 1 (Geng et al. 2012, Kang et al. 2015). In addition, the expression of adhesion molecules belonging to the superfamily of immunoglobulins, such as

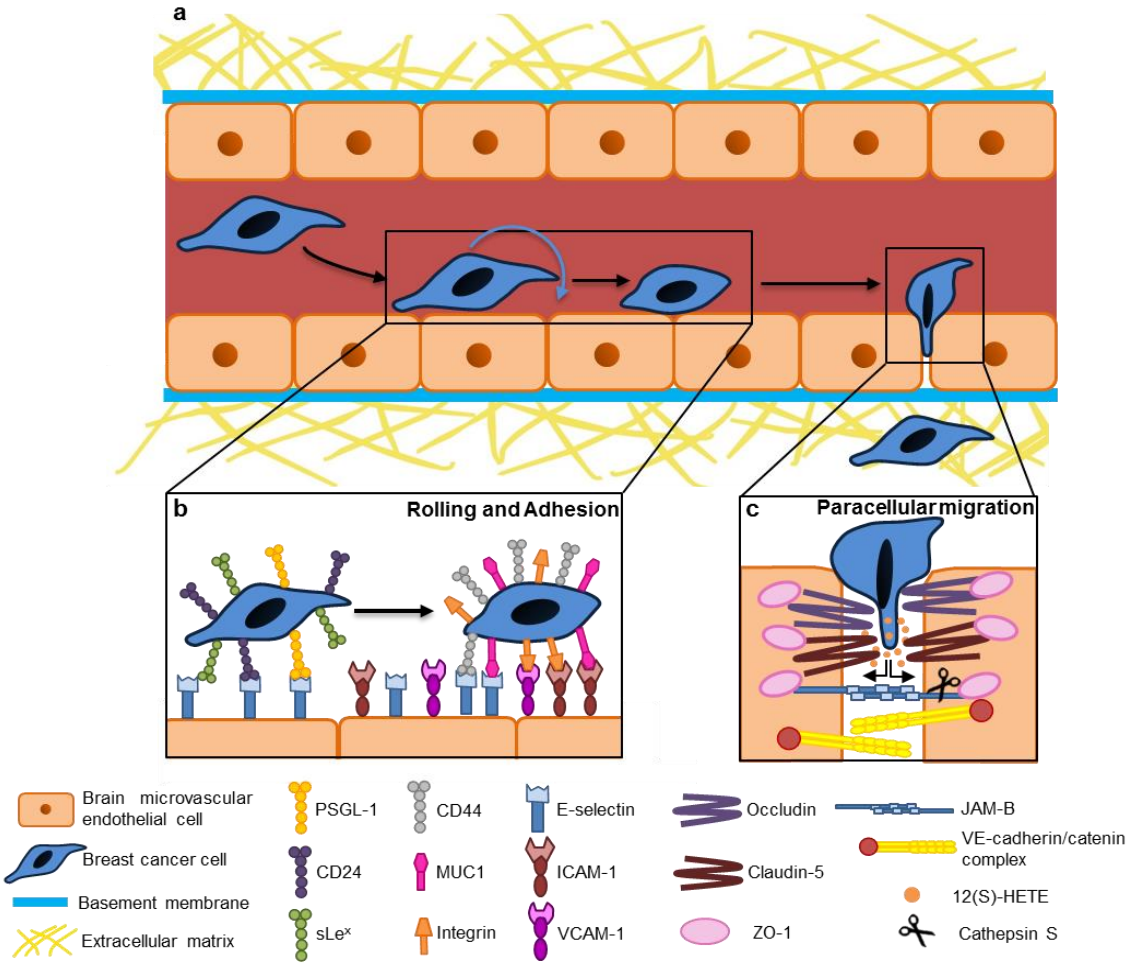


**Fig. 4** Schematic representation of key steps of extravasation of a breast cancer cell (BCC). Rolling, adhesion, and paracellular migration are key steps occurring during metastasization into the brain (a). Rolling and adhesion steps of extravasation allow the tight adhesion between BCC and brain microvascular endothelial cells (BMVECs) in order to facilitate the subsequent migration across the blood-brain barrier (BBB). The elongated tumour cell circulates in the bloodstream. When trapped in small capillaries, the BCC rolls along the endothelium until it finds the best location for crossing the BBB, and attaches to BMVECs. Rolling and adhesion are dependent on pairs of ligands and receptors expressed by both BCC and BMVECs; firstly, BCC expresses ligands, such as platelet selectin glycoprotein ligand-1 (PSGL-1), cluster of differentiation (CD)24, and sialyl Lewis x ($sLe^x$), which interact with endothelial selectin (E-selectin) present in BMVECs surface; during adhesion to brain endothelium, there is an increased expression of CD44 and mucin 1 (MUC1) in BCC, which interact with E-selectin expressed by BMVECs, and integrins that interact with intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1); in addition, MUC1 also binds to ICAM-1 (b). The penetration across the BBB into the brain occurs via paracellular migration, and requires a rearrangement of occludin, claudin-5, and zonula occludens (ZO)-1, and a reduction of junctional adhesion molecule (JAM)-B due to proteolysis by cathepsin S; regarding adhesion junctional proteins, there is a displacement and disruption of vascular endothelial (VE)-cadherin/catenin complex; in addition, BCC secretes 12(S)-hydroxyeicosatetraenoic acid (12(S)-HETE), which promotes BMVECs retraction (c).

intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1), is induced by cytokines and increases on BMVEC membranes (Dufour et al. 1998, Lorger et al. 2011). While ICAM-1 interacts with mucin 1 and integrin αLβ2 (also known as lymphocyte function-associated antigen 1), VCAM-1 binds to integrin α4β1 (also called very late antigen 4) (Geng et al. 2012, Soto et al. 2014). Therefore, all receptor-ligand bonds tightly attach the malignant cell to the BMVEC, and consequently facilitate the migration of the first one.

Although the adhesion process described above has focused on cancer cell-BMVEC interaction, blood components, such as platelets as well as neutrophils, can also facilitate the adhesion of metastatic cells to the brain endothelium. First of all, besides the fact that platelets shield malignant cells against shear forces and/or against recognition and lysis by immune cells (Nieswandt et al. 1999, Palumbo et al. 2005), platelets also have a potential role in promoting the adhesion of BCCs to the brain endothelium (Felding-Habermann et al. 2001). The interaction between cancer cells and platelets leads to activation of integrin αvβ3 in tumour cells, which in turn can interact with integrin αvβ3 highly expressed by BMVECs (Felding-Habermann et al. 2001, Vogetseder et al. 2013). On the other hand, BCCs can establish cell-cell interactions with blood cells, such as neutrophils, even before extravasation (Wu et al. 2001, Lorger and Felding-Habermann 2010). Regarding neutrophils, tumour cells use the adhesion molecules of these blood cells to bind to them, and facilitate the adhesion to the vascular endothelium. Neutrophils express integrin β2 that binds to its ligand ICAM-1 present on membranes of both malignant cells and ECs (Wu et al. 2001, Strell et al. 2007, Lorger et al. 2011). Indeed, neutrophils potentiate the TEM of cancer cells by serving as linking cells, allowing the anchoring of BCCs to the brain vascular endothelium. So, the host cells, such as neutrophils, as well as platelets present in blood circulation are also important for the progression of invasive-metastatic cascade and particularly for adhesion prior to TEM.

The last step of extravasation is the paracellular TEM across the BBB. As mentioned above, cancer cells can cross the blood vessel wall by paracellular and transcellular routes. However, in *in vitro* models of the BBB, the paracellular migration is the most TEM route used by cancer cells to cross the brain endothelium, and consequently the most studied migration route (Rodriguez et al. 2014, Fan and Fu 2016, Vandenhaute et al. 2016). During paracellular migration, metastatic cancer cells interact with endothelial junctions, firstly with TJs and then with AJs, contrarily to what occurs in intravasation. This interaction induces the opening of the intercellular junctions to

allow the squeeze of cancer cells between two ECs, and thus their passage through the endothelial layer. First of all, the firm adhesion of tumour cell to the endothelium induces a partial disruption of endothelial junctions, and finally TEM can completely disrupt these junctions (Fan et al. 2011, Fan and Fu 2016). Regarding TJs, the redistribution of occludin, claudin-5, and ZO-1 leads to a disruption of this type of junctions, and consequently to an increase of BBB permeability (Avraham et al. 2014, Rodriguez et al. 2014). In addition, junctional adhesion molecule-B is proteolysed by cathepsin S, a tumour-derived cysteine protease expressed in early-stages of brain metastases (Sevenich et al. 2014). Concerning AJs, besides the displacement and disruption of VE-cadherin/catenin complex (Lee et al. 2003, Chen et al. 2013), what occurs to these endothelial junctions and their involvement in the brain TEM are still poorly understood. Along with the mentioned mechanisms, the production and secretion of 12(S)-hydroxyeicosatetraenoic acid by BCCs may also induce retraction of BMVECs (Uchide et al. 2007). This morphological alteration of ECs occurs due to a hyperphosphorylation of cytoskeletal proteins such as myosin light chain and actin, and to a rearrangement of integrin αvβ3 (Tang et al. 1993). During paracellular migration, the retraction of BMVECs and the disruption of their intercellular junctions allow cancer cells to incorporate into the EC monolayer, either displacing the ECs or, in some cases, detaching these cells completely from the BM (Hamilla et al. 2014). The hypothesis that cancer cells may also migrate through the endothelial barrier by transcellular TEM remain to be established, but it cannot be discarded. This hypothesis is reasonable, not only because the transcellular pathway is described to intravasation process, but also due to the existence of TJs that connected the BMVECs that may restrict the intercellular transport. However, further studies are required to determine whether this route is used by BCCs to extravasate into the brain.

Regardless of the TEM route used by cancer cells to migrate across the BBB, it remains to be established whether the transmigration of tumour cells leaves the brain endothelium intact or if it destroys intercellular junctions between ECs. Although the endothelial junctions can be disrupted during paracellular migration, it was suggested that malignant cells induce local vessel remodelling characterized by clustering of ECs and their junctions, a similar mechanism to the one observed during TEM of leukocytes (Khuon et al. 2010, Winger et al. 2014). This remodelling of TJs is facilitated by the recruitment of membrane from the endothelial lateral border recycling compartment, a specific compartment containing adhesion molecules, to seal the intercellular gaps at the

exact site of extravasation. Indeed, the absence of any detectable endothelial apoptosis or hypoxia at TEM sites suggests that no significant destruction of the BMVECs occurs during the extravasation process (Lorger and Felding-Habermann 2010). Taken together, this indicates that transmigration of BCCs can occur without causing BBB damage. In contrast, several studies have demonstrated that BCCs can increase the permeability of BMVECs by inducing changes in TJ proteins, such as claudin-5 and ZO-1, and degrading endothelial surface glycocalyx, a structural component of EC monolayer responsible for endothelial integrity (Avraham et al. 2014, Rodriguez et al. 2014, Fan and Fu 2016). These changes are mediated through an increased endothelial secretion of both angiopoietin-2 and tumour necrosis factor alpha, an inflammatory cytokine that also induces angiogenesis through the regulation of angiopoietin-2 expression. In addition, and similarly to the EMT suffered by epithelial cells, ECs may undergo an endothelial-mesenchymal transition (EndMT) (Krizbai et al. 2015). This mechanism leads to loss of endothelial markers and intercellular contacts between adjacent ECs (such as occludin, claudin-5, and VE-cadherin), augment of expression of fibroblast-specific and mesenchymal proteins (including N-cadherin, integrin β1, and fibronectin), and ultimately differentiation into a myofibroblastic phenotype (Medici and Kalluri 2012, Krizbai et al. 2015). The EndMT is stimulated by the TGF-β1 secreted by cancer cells and confers contractility to BMVECs facilitating the TEM of BCCs. So, EndMT is an additional mechanism that allows extravasation of BCCs towards the brain. These findings provide evidence that extravasation into the brain requires mechanisms that are not needed during intravasation.

As in the intravasation, angiogenesis also plays an important role in extravasation. In addition to the reduction of TJ and AJ proteins expression, the overexpression of VEGF and angiopoietin-2 in BMVECs also results in a disruption of intercellular junctions, increasing BBB permeability (Fan et al. 2011, Avraham et al. 2014). This change in permeability is induced by the endocytosis of VE-cadherin, which in turn leads to a disruption of AJs, as well as by structural damage of TJ. Lastly, the disruption of junction-associated protein complexes can also be caused by MMPs released by BCCs, such as MMP-1, -2, and -9 (Wu et al. 2015, Ko et al. 2016). The MMPs also have an important role once cancer cells cross the BMVECs layer, since they degrade the BM. With this step, the BCCs extravasation into the brain is completed.

Since the growth in the primary tumour site until the end of extravasation, numerous obstacles and barriers have been already overcame by the cancer cell. Similarly

to intravasation, extravasation at distant organ sites can also be considered a rate-limiting step of metastatic process, only allowing the passage of a reduced number of malignant cells. Once inside the brain, the microenvironment of this secondary organ plays a crucial role in cell fate decision and, whether malignant cells proliferate, in the subsequent formation of well-established brain metastases.

### 3.2. Proliferation in brain microenvironment

The microenvironment as well as organ-related factors can favour the organ-specific pattern of spread presented by tumour cells, and either promote or supress the proliferation of these cells within the metastatic organ. Stephen Paget was the first one to postulate that the conditions that promote the formation of metastases were not purely anatomical, and hypothesized that the propensity that malignant cells have to spread to specific sites was dependent upon two factors: the cancer cell ('seed') and the receiving organ environment ('soil') (Paget 1889). Later, an alternative theory by James Ewing arose, postulating that mechanical forces and circulatory patterns between the primary tumour and the specific secondary organ played the main role in the metastatic process (Ewing 1928). More recently, a newer hypothesis postulates that cancer cells have the ability to bring their own soil from the primary site – stromal components, such as activated fibroblasts – to the secondary organ (Duda et al. 2010). Despite the existence of several theories to justify the preference that tumour cells have for certain organs, there are still numerous cellular and molecular aspects of the metastatic process that remain a mystery to unravel.

Once inside the brain, BCCs have three possible fates: dormancy, proliferation, or cell death. Cancer cells that enter in a state of dormancy (also designated as quiescence) do not form metastases, not because they undergo apoptosis, but simply because they are in a nonproliferating state (Heyn et al. 2006, Sosa et al. 2014). The dormant malignant cells can reside in the brain for decades until their activation to initiate cancer growth (Chambers et al. 2002, Barkan et al. 2008). The BCCs that adopt this nonproliferating state acquire a perivascular localization, solitary or organized in tiny clusters, through the establishment of a persistent association with the vasculature by using GJ protein Cx43 (Kienast et al. 2010, Ghajar et al. 2013, Stoletov et al. 2013). This intimate connection with the abluminal side of ECs of cerebral capillaries not only allows the survival of dormant malignant cells, but also potentiates the successful proliferation and formation of metastases. Thus, the perivascular localization of cancer cells may probably be a

tumour adaption to acquire an optimal oxygen supply and access to nutrients. The induction of the dormant-to-proliferative metastatic switch is strongly influenced by interactions between the tumour cell and the ECM, and is regulated by signalling pathways, including Src-dependent integrin β1 signalling cascade (Chambers et al. 2002, Barkan et al. 2008). The transition to a proliferative state is associated with drastic reorganization of the cytoskeleton and activation of myosin light chain kinase. Thus, both ECs and ECM may play an active role in regulating tumour dormancy. In addition to dormancy, proliferation and cell death are also cell fates regulated by the balance of signalling pathways, such as PI3K/Akt/mTOR, which inhibits programmed cell death through a prosurvival mechanism (Guo et al. 2015, Molnár et al. 2016). Similarly to dormancy, BCCs can also use Cx43 and L1 cell adhesion molecule, which is expressed by metastatic cells for spreading along brain capillaries and for metastasis outgrowth, to initiate brain metastatic lesion formation in association with vasculature (Stoletov et al. 2013, Valiente et al. 2014). Extravasated cancer cells remain in contact with BMVECs not only because vascular BM can provide the support for metastasis formation prior to the formation of tumour vasculature, called as vessel co-option, but also because BBB protects tumour cells against antitumour immunity and chemotherapeutics drugs (Carbonell et al. 2009, Alkins et al. 2013, Yoshida et al. 2014). Vascular co-option is a mechanism through which the tumour mass obtains an adequate blood supply by leveraging preexisting vessels for growth and dissemination in the brain, independently of sprouting angiogenesis that consists in expansion and remodelling of existing vessels (Leenders et al. 2002, Bugyik et al. 2011). Indeed, the perivascular localization of BCCs allows the interaction with the vascular BM, an active substrate for BCC growth, thus supporting the formation of metastasis prior to the development of the new tumour vessels (Carbonell et al. 2009). For this reason, the ‘soil’ for cancer cells in the CNS has been suggested to be vascular rather than neuronal. This assumption is supported by the extensive brain microvasculature, estimated as 12-20 $m^2$ in the human brain, and by the fact that the brain receives around 20% of cardiac output, despite representing only 2% of body mass (Zlokovic 2011, Abbott 2013). Vascular co-option and angiogenesis have distinct contributions during the initiation of micrometastases, depending not only on the cell numbers in the microtumour, but also on blood supply of local microenvironment (Zhao et al. 2011). In addition to vascular co-option and angiogenesis, aggressive BCCs also have the ability to differentiate into endothelial-like cells, and form vessel-like structures that provide blood supply from the vasculature to hypoxic regions of the tumour

(Qiao et al. 2015, Wagenblast et al. 2015). This capability of cancer cells to develop a vascular phenotype is termed as vascular mimicry. Although in the brain this phenomenon has only been described in glioblastoma (a malignant primary brain tumour) (El Hallani et al. 2010), malignant cell-mediated vascular mimicry plays a vital role in the tumour development independent of angiogenesis. Thus, the anti-angiogenic therapies can be ineffective to treat metastatic brain tumours since cancer cells can adopt survival strategies independent of angiogenesis.

The formation of brain metastasis depends on the reception of multiple inputs from microenvironment by tumour cells, sustaining their survival, migration, and proliferation. The brain possesses a unique microenvironment composed by neurons, astrocytes, microglia, and pericytes (Cardoso et al. 2010, Sá-Pereira et al. 2012), as depicted in Figure 5. Along with BMVECs, all these cells are in dynamic contact, and together form the neurovascular unit. Thus, once inside the brain, proliferating tumour cells must interact with resident cells in order to survive and grow in this secondary organ. Thence, understanding the functional involvement of surrounding cells as well as brain-related factors can be crucial to realize how they contribute to brain metastasis formation.

Neurons transmit information through neurotransmitters, wherein glutamate and γ-aminobutyric acid (GABA) are two of the most available neurotransmitters in the brain responsible for excitatory and inhibitory signalling, respectively (Schousboe et al. 2013). Curiously, BCCs metastasizing into the brain can upregulate the GABAA receptor and GABA transporters, which leads to an increased uptake and metabolization of extracellular GABA as a biosynthetic energy source (Schousboe et al. 2013, Neman et al. 2014). In addition, carcinoma cells can use glutamate decarboxylase to synthetize GABA from glutamate, being this latter neurotransmitter an additional metabolic source for metastatic cancer cells. The acquisition of brain-like properties by BCCs can be a malignant adaptation for these cells to survive, invade and grow into the brain, and thus for brain colonization.

Neurons can establish interactions with glial cells and BMVECs. During brain metastasization, cancer cells induce, directly or indirectly, damage in surrounding neurons (Budde et al. 2012). This damage can be caused by hypoxia, vascular co-option, and excitotoxicity, as a result of cancer proliferation. In addition, malignant cells express calcium-binding protein S100A4, which has a dual role. On one hand, S100A4 promotes the formation of brain metastasis since this metastasis-associated protein participates in a wide range of biological functions, including regulation of angiogenesis, invasion, and

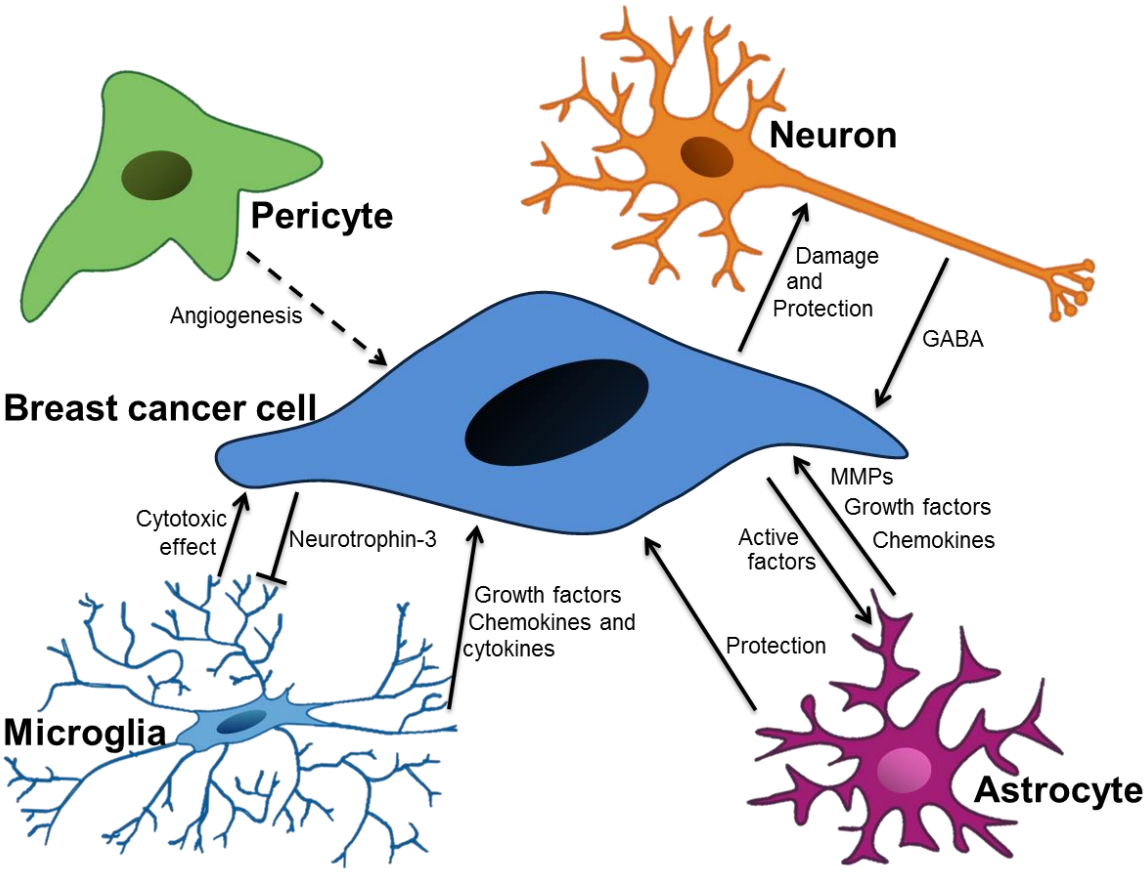


**Fig. 5** Schematic representation of key interactions between breast cancer cell (BCC) and neuron, astrocyte, microglia, and pericyte in brain microenvironment. During the formation of brain metastasis, BCC has a dual role on neuron: either induces damage or produces proteins that protect this cell. In addition, cancer cell has the ability to uptake and metabolize γ-aminobutyric acid (GABA), and use this neurotransmitter as a biosynthetic energy source. Astrocyte interacts with BCC through the production of proteins, such as matrix metalloproteinases (MMPs), growth factors and chemokines that promote metastasis formation. In turn, BCC secretes active factors in order to maintain the astrocytic activity and proteins expression. In addition, astrocyte protects the BCC from apoptosis induced by chemotherapeutic drugs. Microglia has a dual role in BCC, causing cytotoxic effect and producing growth factors, chemokines, and cytokines that are involved in proliferation, angiogenesis, and immune response. In response to the cytotoxic effect of microglia, BCC is able to reduce the number of activated cytotoxic microglia through the secretion of neurotrophin-3. Finally, pericyte has an indirect influence in metastasization, since this cell type is involved in angiogenesis.

cell survival (Boye and Maelandsmo 2010, Dmytriyeva et al. 2012). On the other hand, S100A4 protects neurons from oxidative stress-related death via IL-10R/JAK/STAT3 pathway, a signalling pathway that upregulates antioxidant enzymes and neuroprotective genes in neural cells (Dziennis and Alkayed 2008, Dmytriyeva et al. 2012, Bourgeais et al. 2013). Astrocytes are a type of glial cells that interacts with neurons and has an important role in metastasization. Among the proteins produced by astrocytes are MMPs,

growth factors, and chemokines (Kim et al. 2013, Wang et al. 2013). In turn, tumour cells release active factors, such as interleukin-1β, -6, and -8, to stimulate the production of astrocytic proteins (Xing et al. 2013, Yoshida et al. 2014). In addition to the protection of neurons against toxic substances, reactive astrocytes accumulate around metastatic cells throughout their development into macroscopic metastases (also designated as macrometastases), providing protection to BCCs from apoptosis induced by chemotherapeutic drugs (Lorger and Felding-Habermann 2010, Yoshida et al. 2014). Thence, an effective mechanism to prevent tumour outgrowth and metastasis can pass through blocking the release of substrates by BCCs or surrounding cells into the microenvironment.

Microglia are a cellular type also present in brain microenvironment that have a dual function in formation of brain metastasis. In fact, these neuroglial cells have been shown to exert cytotoxic effects towards cancer cells via production of nitric oxide, a major diffusible mediator that induces death in adjacent cells when in high levels (Brantley et al. 2010). In return to microglia-induced nitric oxide production, BCCs are able to reduce the number of activated microglia releasing neurotrophin-3, a neurotrophic factor for neural survival, development, and plasticity (Louie et al. 2013). On the other hand, the interaction between microglia and BCCs upregulates proteins, including CXCR4, platelet-derived growth factor A (PDGF-A), VEGFs, and cytokines that are involved in regulation of proliferation, angiogenesis, and immune response (Pukrop et al. 2010). Thus, activated microglia are attracted and migrate to brain injury, where the accumulation of these glial cells facilitate the establishment of synapse-like contact with metastatic cancer cells (Fitzgerald et al. 2008, Pukrop et al. 2010). Despite the fact that the role of microglia during the metastatic process is not completely known yet, activated microglia serve as vehicles and guidance cells for the malignant ones into the brain.

Regarding pericytes, these non-ECs intermittently located around the vessel walls are closely related with ECs and enclosed within the BM of microvessels (Sá-Pereira et al. 2012). Among their multiple functions are the maintenance of BBB properties and the regulation of vascular development, stabilization and remodelling. During the formation of new blood vessels, sprouting vascular ECs express PDGF-B to recruit pericytes that express the corresponding surface receptor, PDGF receptor beta (PDGFR-β) (Bergers and Song 2005, Kienast et al. 2010). Thus, this paracrine signalling between the two cell types mediates pericyte recruitment to the vessel wall, being crucial to the proper integrity of structural vasculature. Although knowledge about the involvement of pericytes in brain

metastases is scarce, these cells are also recruited to sparks ECs of new vessels during tumour angiogenesis (Birbrair et al. 2014). Therefore, pericytes may not interact directly with cancer cells, but they promote proliferation and metastasization through angiogenesis. Thus, inhibition of these neurovascular unit cells, induced by nanoparticles or other antiangiogenic agents, may be an effective therapeutic strategy to prevent the angiogenesis-dependent formation of well-established metastasis (Guan et al. 2014).

The crosstalk between malignant cells and resident cells occurs when the first cells arrive in the brain, resulting in the activation of a cascade of different pathways in both tumour and host cells. Furthermore, tumour cells are able to grow in this secondary organ through the gain of ability to exploit the brain endogenous substrates secreted by neighbouring parenchyma cells. The intensive and permanent communication between tumour and host cells promotes the formation of well-established metastasis.

### 3.3. Mesenchymal-epithelial transition and brain colonization

Considerable research effort has been concentrated in understanding the initial steps of the metastatic process, whereas the mechanisms involved in formation of secondary tumours in distal organs are much less understood. This final step of the invasion-metastasis cascade may take years, or even decades, to occur following diagnosis and treatment of the primary tumour, mainly because of dormancy of cancer cells (Witzel et al. 2016). Although the brain microenvironment plays an important role during metastasization by inducing the dormant-to-proliferative metastatic switch, the reacquisition of epithelial phenotype by malignant cells may be the potential mechanism that contributes to the establishment of macrometastases in the brain.

A phenotypic reverse process to the initial EMT that occurs at the primary tumour site, the MET, is an under-investigated mechanism that contributes substantially to the successful colonization of cancer cells at the secondary site (Gunasinghe et al. 2012). During the MET, the reepithelialization of BCCs occurs, i.e., cancer cells reexpress epithelial proteins, such as E-cadherin and pan cytokeratin, and downregulate the expression of mesenchymal proteins, including N-cadherin, vimentin, and Twist (Chao et al. 2010, Yoshida et al. 2014). This phenotypic change promotes the adaption, integration, and survival of metastatic cancer cells at the microenvironment of secondary organ (Chao et al. 2010). Although the induction and regulation of MET in malignant cells are not fully understood, the neurotrophin-3, secreted by tumour cells to reduce the cytotoxic effect of microglia, also plays a key role in the reversion of BCCs to an

epithelial-like state (Louie et al. 2013). In fact, neurotrophin-3 action reduces the migratory ability of carcinoma cells, and consequently facilitates the tumour growth in the brain. Thus, the brain microenvironment, direct or indirectly, appears to play an important role in promoting the survival of metastatic cancer cells via establishment of cell-cell interactions. Curiously, malignant cells can also undergo a partial MET, upregulating epithelial markers, despite maintaining the expression of mesenchymal markers (Chao et al. 2012). The partial MET allows BCCs not only to colonize the brain establishing connections with neighbouring malignant cells via E-cadherin, but also to undergo a second EMT for further dissemination into other cerebral areas. In addition to reexpression of E-cadherin, the expression of GJ proteins Cx26 and Cx43 is increased in brain metastases formed by BCCs, suggesting that the GJs could contribute to MET (Chao et al. 2012). Concerning to signalling, MET is a process dependent on balance between pathways that promote this transition, such as PI3K/Akt signalling, and pathways involved in prevention of MET, including TGF-β signalling (Xue et al. 2012). Therefore, inhibition of MET may contribute to prevent the growth of secondary tumours in BC patients with metastatic diseases.

There are still major gaps in our understanding of the mechanisms behind the process of brain colonization by carcinoma cells, namely on whether the formation of macrometastasis is the result of: 1) successive cell division and genetic instability of cancer cells; or 2) the continued entry of malignant cells into vascular areas where the BBB is compromised, promoted or not by tumour cells that are already within the brain. The first hypothesis is the most likely to occur since *in vivo* studies have demonstrated, through a convenient label detection, that proliferating cells rapidly generate new progenitors through cell divisions, thus possessing stem cell-like characteristics (Kusumbe and Bapat 2009, Stoletov et al. 2013). In addition, the metastatic brain tumour is associated to an alteration in transcription factor genes involved in cellular mechanisms, such as cell growth, differentiation, and cellular reprogramming via MET (Jeevan et al. 2016). Furthermore, astrocytes present in brain microenvironment also contribute to tumour cells growth, promoting not only the entry in S-phase of cell cycle, the phase responsible for the DNA replication, but also the self-renewal of BCCs, a process that gives rise to indefinitely more cells of the same cell type (Xing et al. 2013, Jeevan et al. 2016). Thus, MET together with the cerebral microenvironment appear to play a critical role in the development of macrometastasis in the brain.

As during BC growth, invasion, intravasation, and extravasation, angiogenesis also plays a pivotal role during secondary tumour growth. Once malignant cells extravasate into the brain, these cells usually remain in close contact with vascular ECs, and have the ability to obtain blood supply only through vascular co-option (Lorger and Felding-Habermann 2010). When the secondary tumour mass grows and reaches a macroscopic size, vessel co-option is followed by vessel regression and stimulation of angiogenesis, which shall provide the nutrients and oxygen to the mass of metastatic tumour cells (Holash et al. 1999). The enhancement of angiogenesis is induced by the significantly increased production of VEGF-A and angiopoietin-2 by BCCs (Kim et al. 2004, Avraham et al. 2014). In fact, brain lesions present lower vascular permeability, but more angiogenesis compared to primary tumours (Monsky et al. 2002). Thus, the mechanisms behind the formation of new blood vessels and/or recruitment of preexisting vessels appear to be strongly dependent on tumour cell origin as well as on the brain microenvironment. Although angiogenesis is believed to be crucial for the growth of brain metastatic tumours, carcinoma cells can survive and proliferate without the formation of new vessels. In some cases, BCCs proliferate within small blood vessels and grow intravascularly (Lu et al. 2007). Their continuous growth leads to disruption of the BBB, and ultimately to thrombosis-like infarction of the brain parenchyma. In addition, the malignant cells localised along the vessels whose barrier property is compromised migrate to distant sites to maintain protected by an intact BBB (Lu et al. 2007). Curiously, tumour cells adhere directly to the BM of the capillary, replacing the astrocytes from the basal surface, but remaining the original structure of incorporated vessels. Hence, the carcinoma cells have the ability to adapt to the brain microenvironment, distributing in ideal sites where they can receive nutrients and oxygen to promote their proliferation, and hence form brain metastasis.

The formation of robust macrometastasis represents the endpoint of the multistep metastatic process. Only a small minority of cancer cells, the most metastatic ones, have the ability to overcome all the obstacles that normally prevent the formation of secondary tumours. Therefore, a successful metastatic tumour cell is able to pass through the growth *in situ*, invasion of mammary duct, migration toward a blood vessel wall, intravasation into the bloodstream, survival during the course from the primary to secondary site, extravasation across vascular endothelium barrier, establishment of interactions with microenvironment cells, and finally formation of metastasis. During all the invasive-metastatic cascade, reciprocal interactions between tumour and host cells play a pivotal

role, allowing the adaptation of malignant one to the different microenvironment in which the cell passes, and thus promoting the metastasization of the 'target organ'. So, despite the already developed studies allow to understand and unravel, even partially, the mechanisms of the metastatic steps that culminate in well-establishment of metastasis, more studies are needed to develop novel therapeutic approaches able to prevent the formation of these growths at distant organs.

## 4. Conclusion

Over the past decades, a substantial improvement in the diagnosis and treatment of BC patients, including those who evolve to a metastatic stage, has been achieved. One exception respects to the patients with brain metastasis for whom there is still no efficient treatment. Since brain metastasization is a major clinical problem and a leading cause of death from cancer, it is pivotal a better knowledge of the pathways that orchestrate the invasion-metastasis cascade. However, compared to other 'target organs' of BCCs, such as the bone, the mechanisms responsible for CNS metastasis formation remain poorly understood and substantially less frequently investigated. In addition, the existence of the BBB, the capacity of cancer cells to migrate to sites where the endothelium barrier remains intact, and the protection provided to malignant cells by host cells render difficult the development of an optimal strategy for the treatment of patients with brain metastasis. One important strategy may involve the prediction of occurrence of brain metastasization through the discovery of new biomarkers and genetic signatures, and/or the comprehension of cellular behaviour of cancer cells that are in circulation or even in primary tumour site. This way, in addition to overcoming the BBB for the transport of therapeutic agents into the brain, this life-threat outcome for BC patients might become early detected and effectively prevented. Moreover, understanding of the contribution of interactions between metastatic BCCs and host cells, especially ECs, to successful brain colonization may also provide other therapeutic approach to prevent or decrease the formation of brain metastasis. Since the roles played by host cells during the establishment of metastasis are rapidly beginning to be appreciated, this strategy can be crucial to develop specific therapies to prevent the brain metastasis formation. Therefore, although enormous challenges remain, it is anticipated that the continuous research in brain metastasization area will culminate into novel clinical approaches.

# 5. Aims

The exact mechanisms, including the time at which they occur and their intervenients, involved in brain metastasization of BC still need to be elucidated, mainly those that occur once malignant cells cross the BBB. Therefore, with this project we aimed to: (1) establish the temporal profile of BCCs metastasization to the brain and characterize the metastasizing cells phenotype, (2) investigate the BBB properties and vascular events along the process of BC metastasization to the brain, and (3) assess signalling mechanisms involved in attraction of malignant cells into the brain and proliferation in the nervous tissue. To this end, we used female mice inoculated with 4T1 BCCs sacrificed at 5 hours, 3 days, 7 days or 10 days and mice injected with vehicle (control) sacrificed at 5 hours to profile the alterations of BBB and brain parenchyma along the metastatic progress, to establish the temporal sequence of these alterations, and to relate them with the appearance of brain metastases. Thus, we will contribute to clarify the time-course and cellular and molecular players behind the establishment of brain metastasis of BC.

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# Chapter II

## Capturing changes in the blood-brain barrier and brain parenchyma during brain metastasization of breast cancer

## Abstract

Brain metastasization of breast cancer is a multistep process dependent on the attraction of cancer cells to the brain endothelium and their posterior transendothelial migration by still unclear pathways. This study was designed to characterize the process of brain metastasization in terms of the phenotype of malignant cells, the signaling molecules involved, and their relationship with the blood-brain barrier (BBB) disruption. Therefore, we outlined to: i) establish the temporal profile of breast cancer cells metastasization to the brain and characterize the metastasizing cells phenotype, ii) investigate the vascular events and BBB properties along the process of BC metastasization to the brain, and iii) assess signalling mechanisms involved in attraction of malignant cells into the brain and proliferation in the nervous tissue. We examined cerebella, cranial hippocampi, and striata of female mice inoculated with 4T1 breast cancer cells along the process of brain metastasization (5 hours, 3 days, 7 days, and 10 days) and of female mice injected with vehicle (control; sacrificed at 5 hours) by conventional haematoxylin and eosin staining, and by immunohistochemical and immunofluorescence analysis. Brain metastasization resulted in the occurrence of well-established metastasis along time, in entrance of malignant cells as mesenchymal cells into the brain and then the acquisition of epithelial markers by these cells, as well as the decrease of claudin-5 expression by brain microvascular endothelial cells, and the entrance of thrombin in brain parenchyma. In addition, hypervascularization in cranial hippocampus appeared to be associated to brain colonization. Brain metastasization also resulted in the increase of platelet-derived growth factor B expression by tumour cells. The occurrence of cysteine-X amino acid-cysteine receptor 4 (CXCR4)-positive cells increased in early stages of the process of brain metastasization, and then the number stabilized in later stages of this process. Taken together, these results highlight the contribution of BBB disruption, brain parenchyma alterations, signalling molecules, and phenotypic transition of tumour cells along the process of brain metastasization of breast cancer.

*Keywords*: Blood-brain barrier disruption; breast cancer brain metastasis; extravasation; mesenchymal-epithelial transition; microvasculature.

## Resumo

A metastização cerebral do cancro da mama é um processo com múltiplos passos dependente da atracão das células cancerígenas para o endotélio cerebral e da sua posterior migração através de vias ainda pouco conhecidas. Este estudo foi desenhado para caracterizar o processo de metastização em termo de fenótipo das células malignas, das moléculas sinalizadoras envolvidas e a sua relação com a disrupção da barreira hematoencefálica (BHE). Por este motivo, nós delineámos: i) estabelecer o perfil temporal da metastização por parte das células cancerígenas da mama para o encéfalo e caracterizar o fenótipo das células metastáticas, ii) investigar as alterações vasculares e as propriedades da BHE ao longo do processo de metastização do cancro da mama para o encéfalo, e iii) aceder aos mecanismos de sinalização envolvidos na atração de células malignas para o encéfalo e na proliferação no tecido nervoso. Nós examinámos cerebelos, hipocampos craniais e estriados de ratinhos fêmea inoculados com células cancerígenas da mama 4T1 ao longo do processo de metastização cerebral (5 horas, 3 dias, 7 dias e 10 dias) e de ratinhos fêmea injetados com veículo (controlo; sacrificados às 5 horas) através da coloração convencional de hematoxilina e eosina e através de análise imuno-histoquímica e imuno-fluorescente. A metastização do encéfalo resultou no aparecimento de metástases bem estabelecidas ao longo do tempo, na entrada de células malignas expressando o seu fenótipo mesenquimal para dentro encéfalo e posterior aquisição de marcados epiteliais, assim como na diminuição da expressão de claudina-5 nas junções das células endoteliais microvasculares cerebrais, e na entrada de trombina no parênquima encefálico. Para além disso, o aumento da vascularização no hipocampo cranial aparentou estar associado à colonização desta região pelas células cancerígenas da mama. A metastização do encéfalo também resultou no aumento da expressão do fator de crescimento B derivado das plaquetas pelas células tumorais. O número de células positivas para o recetor CXCR4 aumentou nos estadios iniciais do processo de metastização do encéfalo e, em estadios mais avançados deste processo, esse número estabilizou. Assim, estes resultados realçam a contribuição da disrupção da BHE, das alterações no parênquima encefálico, das moléculas sinalizadoras e da transição fenotípica das células tumorais ao longo do processo de metastização do cancro da mama para o encéfalo.

*Palavras-chave*: Disrupção da barreira hematoencefálica; extravasação; metástases cerebrais do cancro da mama; micro-vasculatura; transição mesenquimal-epitelial.

# 1. Introduction

Breast cancer (BC) represents the second most frequent cause of central nervous system (CNS) metastases, after lung cancer, with these secondary tumours occurring in 12-31% of patients (Vuong et al. 2011, Saha et al. 2013). Brain metastasis is commonly associated with poor prognosis and diminished quality of life, being normally a catastrophic life-threat outcome for patients (Cruz-Muñoz and Kerbel 2011, Jaboin et al. 2013). Unfortunately, there are no targeted therapies specific for this secondary tumour formation (Steeg et al. 2011), and the cellular and molecular events underlying their establishment and progression are poorly understood. However, it is known that the metastatic cascade comprises several steps, such as the epithelial-mesenchymal transition (EMT) in mammary duct, entrance of cancer cells into bloodstream (intravasation), arrest in brain capillaries, the extravasation into the 'target organ', and the mesenchymal-epithelial transition (MET) that together contribute for a successful colonization of the brain by cancer cells (Wyckoff et al. 2000, Stoletov et al. 2010, Gunasinghe et al. 2012, Videira et al. 2014). The arrival of viable BC cells (BCCs) to the brain can result from a trapping in small vessels driven by organ-derived chemoattractants that play an important role in the homing of carcinoma cells into the brain (Hujanen and Terranova 1985, Lee et al. 2004, Salmaggi et al. 2014). The unique properties of the brain microvasculature, which forms the blood-brain barrier (BBB), selectively regulate the entrance of molecules and cells into the brain parenchyma through elaborate junctional complexes, including tight junctions (TJs) and adherens junctions (Cardoso et al. 2010). Despite the BBB restricted permeability, the most metastatic cells are able to extravasate into this privileged organ regarding the appearance of metastases. The extravasation of BCCs across the BBB involves rolling, firm adhesion of malignant cells to the brain microvascular endothelial cells (BMVECs), followed by transendothelial migration (TEM) via paracellular route (Lorger and Felding-Habermann 2010, Stoletov et al. 2010). During paracellular migration, the interaction between metastatic cells and BMVECs induces the opening of the junctions to allow the squeeze of cancer cells between two ECs, and thus leads to BBB disruption (Fan et al. 2011, Fan and Fu 2016). Once inside the brain, the colonization of this secondary organ by BCCs requires not only a phenotypic transition whereby malignant cells reacquire epithelial characteristics, but also angiogenesis to allow an optimal oxygen supply and access to nutrients, favouring

the formation of well-established brain metastasis (Zhao et al. 2011, Gunasinghe et al. 2012).

In this study, we wanted to understand the cellular and molecular events involved in BBB and brain parenchyma alterations that are prevalent during the brain metastasization. The results presented here reveal the time-course and interdependence of BBB breach, phenotypic alterations of malignant cells, and signalling events along the attraction to the BMVECs and brain metastases formation. Moreover, by establishing the temporal sequence of events occurring in brain metastasization and identifying the early ones, this study contributes to an in-depth understanding of the malignant behaviour and points to potential therapeutic targets for modulation in order to prevent the extravasation and brain colonization processes, essential to developed novel strategies to improve the life expectancy of BC patients.

# 2. Materials and methods

## 2.1. Cell culture and animals

Murine mammary carcinoma 4T1 cells, an aggressive metastatic triple-negative BCC line, were purchased from ATCC (Middlesex, UK), and maintained in RPMI 1640 medium (PAN Biotech, Aidenbach, Germany) supplemented with Ultraglutamine I (Lonza, Basel, Switzerland) and 5% heat-inactivated fetal bovine serum (PAN Biotech) in a 5% $CO_2$ atmosphere at 37 ºC. Female BALB/c mice at the age of 7-8 weeks were purchased from Charles River Laboratories (Wilmington, MA, USA), and were housed and bred in the animal facility of the Biological Research Centre of the Hungarian Academy of Sciences, Hungary. The xenographs of metastatic breast cancer (n=4) were developed by inoculating, under isoflurane anaesthesia, 1 x $10^6$ 4T1 cells in the right common carotid artery in a total volume of 200 µL of Ringer-HEPES, whereas control mice (n=2) were inoculated with vehicle. At 5 hours, 3, 7, or 10 days post-tumour cell inoculation and at 5 hours post-vehicle injection, brain tissue was harvested after perfusing deeply anesthetized animals with 50 mL of phosphate-buffered saline (PBS), followed by 25 mL of 4% paraformaldehyde in PBS. Brains were post-fixed overnight in 4% paraformaldehyde in PBS at 4 ºC and kept in PBS.

All procedures were conducted according to the recommendations of the Declaration of Helsinki and Tokyo, and were performed according to the EU Directive 2010/63/EU on the protection of animals used for experimental and other scientific purposes. The protocol was reviewed and approved by the Regional Animal Health and Food Control Station of Csongrád County (licence number: VI-I-001/2980-4/2012) of the Hungarian Academy of Sciences. Fixed brain samples were shipped to the Faculty of Pharmacy, University of Lisbon, Portugal, where the microscopic analysis was performed.

## 2.2. Histology

Brain processing, paraffin embedded, and sectioning were performed at the Histology and Comparative Pathology Laboratory at Instituto de Medicina Molecular, headed by Doctor Tânia Carvalho. Mouse brains were serially cut into 4-µm-thick sections to performed the stainings and labellings described below. To establish the brain regions to be analysed, a preliminary set of experiments was performed in which the brains were coronally cut in five regions according to the following bregma coordinates:

medulla, -8.24 mm; cerebellum, -6.12 mm; caudal hippocampus, -3.52 mm; cranial hippocampus, -1.82 mm; and striatum, 0.5 mm. Analysis of the percentage of metastases formation by staining with haematoxylin and eosin (H&E), as described below, showed that cranial hippocampus and striatum were two brain regions with high percentage of brain metastases at later stages, that cerebellum is the brain region that presents secondary tumours only 10 days post-tumour cell inoculation, and that the medulla is the only brain region that does not present any metastasis along time (Table 1). Based on these results, the cerebellum, cranial hippocampus, and striatum were chosen for further analysis.

**Table 1** Percent distribution of metastases in each brain region of mice injected with 4T1 cells along time.

| | Brain region | | | | |
|---|---|---|---|---|---|
| **Timepoint** | **Medulla** | **Cerebellum** | **Caudal hippocampus** | **Cranial hippocampus** | **Striatum** |
| **5 hours** | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| **3 days** | 0.0 | 0.0 | 50.0 | 0.0 | 50.0 |
| **7 days** | 0.0 | 0.0 | 29.3 | 28.9 | 41.8 |
| **10 days** | 0.0 | 9.3 | 25.8 | 33.5 | 31.4 |

### 2.3. Reagents and antibodies

Xylene and Quick-D Mounting Medium were purchased from Klinipath (Duiven, Netherlands). Papanicolaou's solution 1a Harris' haematoxylin solution, Y-solution 0.5% alcoholic, and Triton X-100 were obtained from Merck Millipore (Darmstadt, Germany). Bovine serum albumin (BSA), goat serum, and Tris borate-EDTA were acquired from Sigma-Aldrich (St. Louis, MO, USA). Citric acid was purchased from Chem-Lab (Zedelgem, Belgium). 4',6-diamidino-2-phenylindole (DAPI) and SlowFade® Diamond Antifade Mountant were obtained from Thermo Fisher Scientific (Waltham, MA, USA). All other chemicals were of analytical grade.

Mouse monoclonal anti-claudin-5 (4C3C2), rabbit polyclonal anti-CXCR4/CD184, rabbit polyclonal anti-N-cadherin/CDH2, mouse monoclonal anti-pan Cytokeratin (C-11), rabbit polyclonal anti-PDGF-B, rabbit polyclonal anti-vimentin, goat anti-rabbit Alexa Fluor® 555, goat anti-mouse Alexa Fluor® 647, and SuperPicture™ Polymer Detection Kit, DAB, broad spectrum polymer were from Thermo Fisher Scientific. Fluorescein-conjugated *Lycopersicon esculentum* (tomato) lectin was obtained

from Vector Laboratories (Burlingame, CA, USA). Mouse monoclonal anti-thrombin (F-1) was acquired from Santa Cruz Biotechnology (Burlingame, TX, USA).

### 2.4. Haematoxylin and eosin staining

For H&E staining, the tissue was deparaffinised in xylene (10 minutes), rehydrated in successive ethanol solutions (100% ethanol for 20 seconds, 96% ethanol for 10 seconds, and 70% ethanol for 10 seconds), and in tap water (10 seconds). The nuclei were stained with Papanicolaou's solution 1a Harris' haematoxylin solution for 4 minutes. Sections were then differentiated using a solution of 1% hydrochloric acid in 70% ethanol (5 seconds), and bluing in 1% ammonia water (5 seconds). The cytoplasm was stained with eosin Y-solution 0.5% alcoholic for 10 minutes. Finally, sections were dehydrated, and mounted with Quick-D Mounting Medium.

### 2.5. Immunofluorescence and immunohistochemistry

Tissue sections were labelled for claudin-5, cysteine-X amino acid-cysteine receptor 4 (CXCR4), pan Cytokeratin, thrombin, tomato lectin, and vimentin by immunofluorescence (IF), or stained for neuronal cadherin (N-cadherin), and platelet-derived growth factor B (PDGF-B) by immunohistochemistry (IHC), as described below.

For IF labelling, tissue sections were deparaffinised in xylene (20 minutes), and rehydrated through successive immersion in 100% ethanol (20 minutes), 96% ethanol (10 minutes), 70% ethanol (10 minutes), and finally tap water (10 minutes). A 3% hydrogen peroxide solution was used to inhibit endogenous peroxidase activity, for 30 minutes. Heat-mediated antigen retrieval was performed with 10 mM citrate buffer pH 6.0 during 15 minutes for sections to be labelled for claudin-5, CXCR4, pan Cytokeratin, tomato lectin, and vimentin, or with 10 mM Tris-borate EDTA buffer pH 9.0 during 10 minutes for sections to be labelled for thrombin. A permeabilization step was performed with 0.5% Triton X-100 for 15 minutes (with the exception of sections for thrombin), and tissue sections were blocked with 3% BSA during 60 minutes. The primary antibodies were diluted in 3% BSA, 0.5% Triton X-100 solution, and sections were incubated overnight at 4 ºC. Then, the incubation with the respective fluorescent-labelled secondary antibody (anti-rabbit Alexa Fluor® 555, or anti-mouse Alexa Fluor® 647) diluted in 3% BSA were performed during 60 minutes at room temperature, as summarized in Table 2. After the antigen retrieval treatment, washes were performed using PBS three times for 10 minutes between all the steps. Negative controls with omission of primary antibodies were

performed to exclude nonspecific binding or cross reactivity. Nuclei were labelled with DAPI diluted 1:1000 in PBS for 2 minutes, followed by slides mounting with SlowFade® Diamond Antifade Mountant.

**Table 2** Summary of the antibodies and experimental conditions used for immunofluorescence and immunohistochemical analysis.

| Technique | Marker | Primary antibody | Dilution | Secondary antibody | Dilution |
|---|---|---|---|---|---|
| IF | **Claudin-5** | Thermo Fisher Scientific, #35-2500, Mouse Mc | 1:500 | Alexa Fluor® 647 Thermo Fisher Scientific, #A-21235 Goat anti-mouse | 1:500 |
| | **CXCR4** | Thermo Fisher Scientific, #PA3-305, Rabbit Pc | 1:100 | Alexa Fluor® 555 Thermo Fisher Scientific, #A-21428 Goat anti-rabbit | 1:500 |
| | **pan CK** | Thermo Fisher Scientific, #MA5-12231, Mouse Mc | 1:100 | Alexa Fluor® 647 Thermo Fisher Scientific, #A-21235 Goat anti-mouse | 1:500 |
| | **Tomato lectin** | Vector Laboratories, #FL-1171 | 1:400 | NA | NA |
| | **Thrombin** | Santa Cruz Biotechnology, #sc-271449, Mouse Mc | 1:200 | Alexa Fluor® 647 Thermo Fisher Scientific, #A-21235 Goat anti-mouse | 1:500 |
| | **Vimentin** | Thermo Fisher Scientific, #PA5-27231, Rabbit Pc | 1:100 | Alexa Fluor® 555 Thermo Fisher Scientific, #A-21428 Goat anti-rabbit | 1:500 |
| IHC | **N-cadherin** | Thermo Fisher Scientific, #PA5-19486, Rabbit Pc | 1:100 | HRP from SuperPicture™ Polymer Detection Kit Thermo Fisher Scientific, #87-9663 | NA |
| | **PDGF-B** | Thermo Fisher Scientific, #PA1-27394, Rabbit Pc | 1:75 | HRP from SuperPicture™ Polymer Detection Kit Thermo Fisher Scientific, #87-9663 | NA |

CK, cytokeratin; CXCR4, cysteine-X amino acid-cysteine receptor 4; HRP, horseradish peroxidase; IF, immunofluorescence; IHC, immunohistochemistry; Mc, monoclonal; NA, not applicable; N-cadherin, neuronal cadherin; Pc, polyclonal; PDGF-B, platelet-derived growth factor B.

For IHC staining, tissue sections were deparaffinised in xylene (20 minutes), and rehydrated through successive immersion in 100% ethanol (20 minutes), 96% ethanol (10 minutes), 70% ethanol (10 minutes), 50% ethanol (10 minutes), and finally tap water (10 minutes). A 3% hydrogen peroxide solution was used to inhibit endogenous peroxidase activity, for 30 minutes. Heat-mediated antigen retrieval was performed with 10 mM citrate buffer pH 6.0 during 15 minutes. The tissue sections stained for PDGF-B were blocked with a 3% BSA, 0.5% Triton X-100 solution and sections stained for N-cadherin were blocked in a 5% goat serum, 1% TritonX-100 solution for 60 minutes. The primary antibodies were both diluted in the respective blocking solutions, at concentrations indicated in Table 2, and sections were incubated overnight at 4 ºC. Then, the SuperPicture™ Polymer Detection Kit was used according to the manufacturer's instructions to detect positive staining, followed by development with 3,3'-diaminobenzidine tetrahydrochloride (DAB) for 1 minute for N-cadherin staining or for 2 minutes for PDGF-B staining. After the antigen retrieval treatment, washes were performed using PBS three times for 10 minutes between all the steps. For N-cadherin, washes after the incubation with primary antibody were performed with PBS, 1% Triton X-100 solution, also three times for 10 minutes. Negative controls with omission of primary antibodies were performed to exclude nonspecific binding or cross reactivity. Haematoxylin counterstaining was performed only for N-cadherin immunostaining. Finally, sections were dehydrated, and mounted with Quick-D Mounting Medium.

### 2.6. Data analysis

For the analysis of the metastases distribution, the brain metastases of each brain region (the medulla, the cerebellum, the caudal hippocampus, the cranial hippocampus, and the striatum) at each timepoint were counted manually by a single observer. The number of metastases per region was presented as percentage of the total number of metastases detected in the entire brain at each timepoint.

Photographs of H&E and IHC staining were acquired on bright field microscope (Olympus, model BX51) with an integrated digital camera (Olympus, model DP50) and photographs of IF labelling were acquired on confocal microscope (Leica, model TCS SPE), equipped with 3 lasers (488, 532, and 635 nm). Ten fields of the cerebellum, cranial hippocampus, and striatum of each animal were analysed using the ImageJ 1.29x software (National Institutes of Health, USA), as detailed below.

Metastases appearance and development were analysed based on the evaluation of total tumour area determined by delimitation of each metastasis per field, and results were expressed in $\mu m^2$ of tumour area. For evaluation of malignant cells entrance into the brain parenchyma, the number of individual vimentin-positive cells inside or near a blood vessel per field was measured. Moreover, the formation of brain metastasis was also analysed based on the evaluation of the number of vimentin-positive cells per field. To evaluate the phenotypic changes of tumour cells, the malignant cells were classified according to their phenotype in vimentin-positive/pan Cytokeratin-negative, vimentin-positive/pan Cytokeratin-positive, and vimentin-negative/pan Cytokeratin-positive, and the number of cells expressing the respective phenotype per field was counted. For evaluation of TJ protein claudin-5 immunoreactivity in ECs, each blood vessel was delimited, the vascular immunoreactivity was measured, and results were expressed by the mean intensity of claudin-5 per $\mu m^2$ of blood vessel. To evaluate the entrance of thrombin into the brain parenchyma, an indicator of BBB hypermeability (Janota et al. 2015), the total number of thrombin deposits per section was measured. In addition, vascular parameters were analysed based on the TJ marker claudin-5. To evaluate the microvascular density, the area of claudin-5-positive blood vessels per field was measured (Brito et al. 2013), and data was expressed as total area of blood vessels per $\mu m^2$ of brain tissue. To analyse the number of cells expressing of PDGF-B, the number of PDGF-B-positive cells per field was counted. Moreover, the number of cells expressing CXCR4 was evaluated by measuring the number of CXCR4-positive cells per field.

### 2.7. Statistical analysis

Results were analysed using GraphPad Prism® 5.0 (GraphPad Software, San Diego, CA, USA), and are expressed as mean±SEM. One-way ANOVA and the Dunnett post hoc test were used to compare how the parameters evolved in 4T1 and vehicle injected mice at each timepoint, for each brain region separately. In addition, one-way ANOVA and the Bonferroni post hoc test were used to evaluate how the parameters evolved in 4T1 injected mice groups along time for each brain region separately and to determine whether there were statistically significant differences between brain regions. P values less than 0.05 were considered statistically significant.

# 3. Results

## 3.1. Brain metastases occur from 7-days onwards after inoculation

To monitor metastases appearance and development, the tumour area was determined for cerebellum, cranial hippocampus and striatum of 4T1 injected mice along time (Fig. 1). Observation of H&E stained sections revealed that well-established metastasis occurred at 7 days in all three brain regions, which augmented thereafter (Fig. 1a, b, and c). There was a ∼3-fold or higher increase of the tumour area from 7 to 10 days in all three brain regions (Fig. 1d, e, and f). In line with the preliminary set of experiments (Table 1), the cerebellum was the least affected region. In fact, the cerebellum had four times less tumour area at 7 days compared to cranial hippocampus, even though not significant, and almost three times less at 10 days ($P<0.01$). Thus, these results show that the appearance of brain metastasis with larger dimensions (or macrometastases) occurs from 7-days onwards after the inoculation of breast tumour cells.

## 3.2. Malignant cells undergo a mesenchymal-epithelial transition

Having established the profile of brain metastases occurrence, we further wanted to identify individual tumour cells and establish if they cross the BBB in their mesenchymal phenotype and maintain this phenotype until brain colonization, when malignant cells undergo the MET. For these purposes, we analysed the time-dependent expression of widely used mesenchymal (N-cadherin and vimentin) and epithelial (tomato lectin and pan Cytokeratin) markers, alone or in combination (Fig. 2-5).

The IHC analysis of N-cadherin showed the presence of individual N-cadherin-positive cells already at 5 hours post-tumour cell inoculation, and that these cells were normally localized near blood vessels in all brain regions (Fig. 2a, b, and c). Observation of N-cadherin staining along time suggests an increase of the number of positive-cancer cells at 3 days in all brain regions compared to 5 hours. At 7 and 10 days, the BCCs that composed macrometastases did not express N-cadherin, whereas individual cells still express the mesenchymal marker, as observed in the cerebellum at 7 days (Fig. 2a). These results suggest that malignant cells extravasate into the brain as mesenchymal cells, and that once inside this secondary organ, they lose the mesenchymal characteristics as brain metastases are formed.

The vimentin and tomato lectin double labelling IF analysis allowed the observation of cancer cells presenting a mesenchymal phenotype located inside or near a

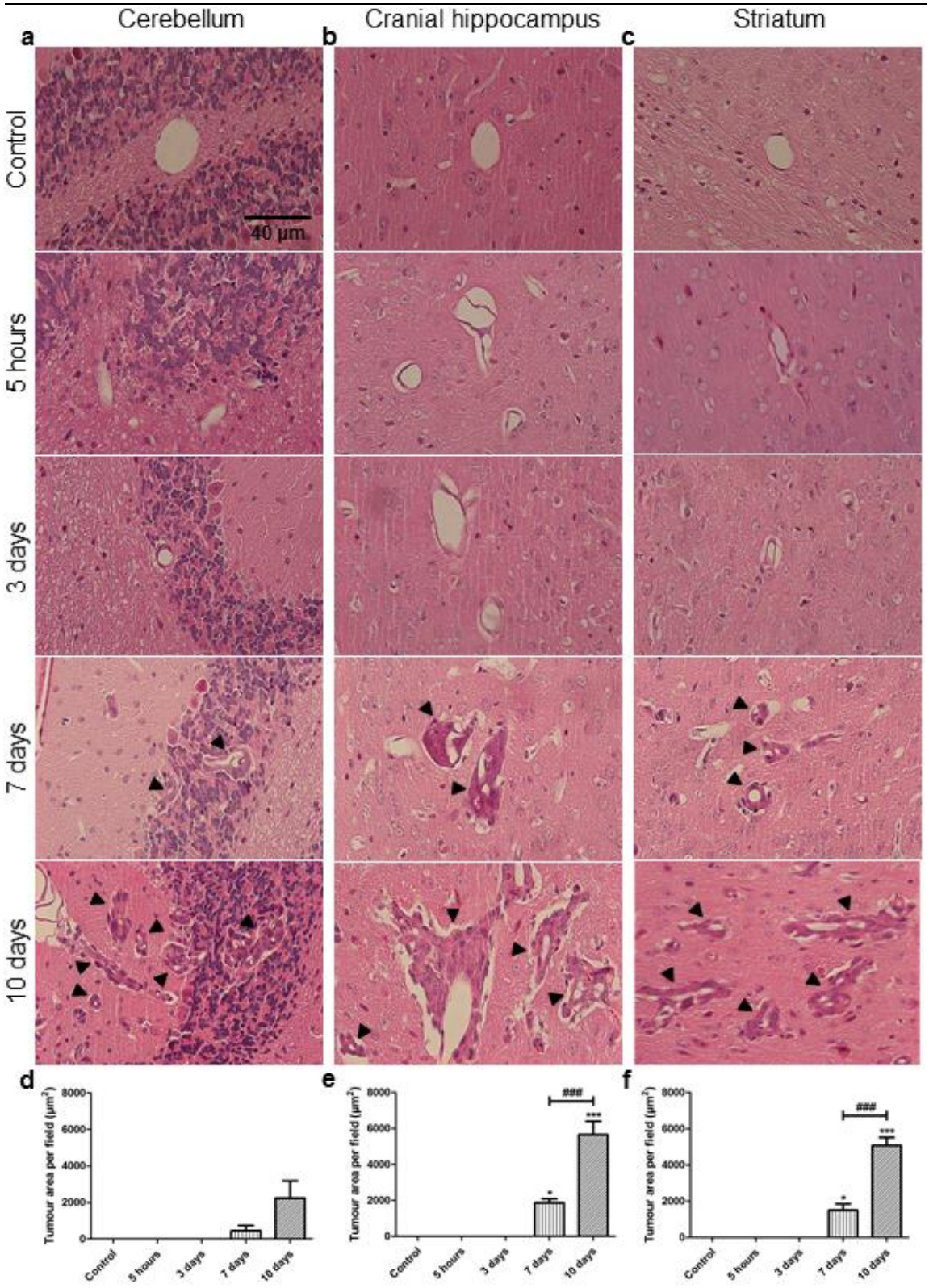


**Fig. 1** Tumour area occupied by breast cancer cells along the process of brain metastasization. Cerebellum, cranial hippocampus, and striatum sections of control mice and of mice at different times post-tumour cell inoculation were processed for haematoxylin and eosin analysis. Representative immunohistological patterns of the area occupied by brain metastases indicated by arrowheads in cerebellum (a), cranial hippocampus (b), and striatum (c). Semi-quantitative analysis of the tumour area per field in cerebellum (d), cranial hippocampus (e), and striatum (f). *P<0.05, ***P<0.001 vs. control; ###P<0.001 between indicated groups.

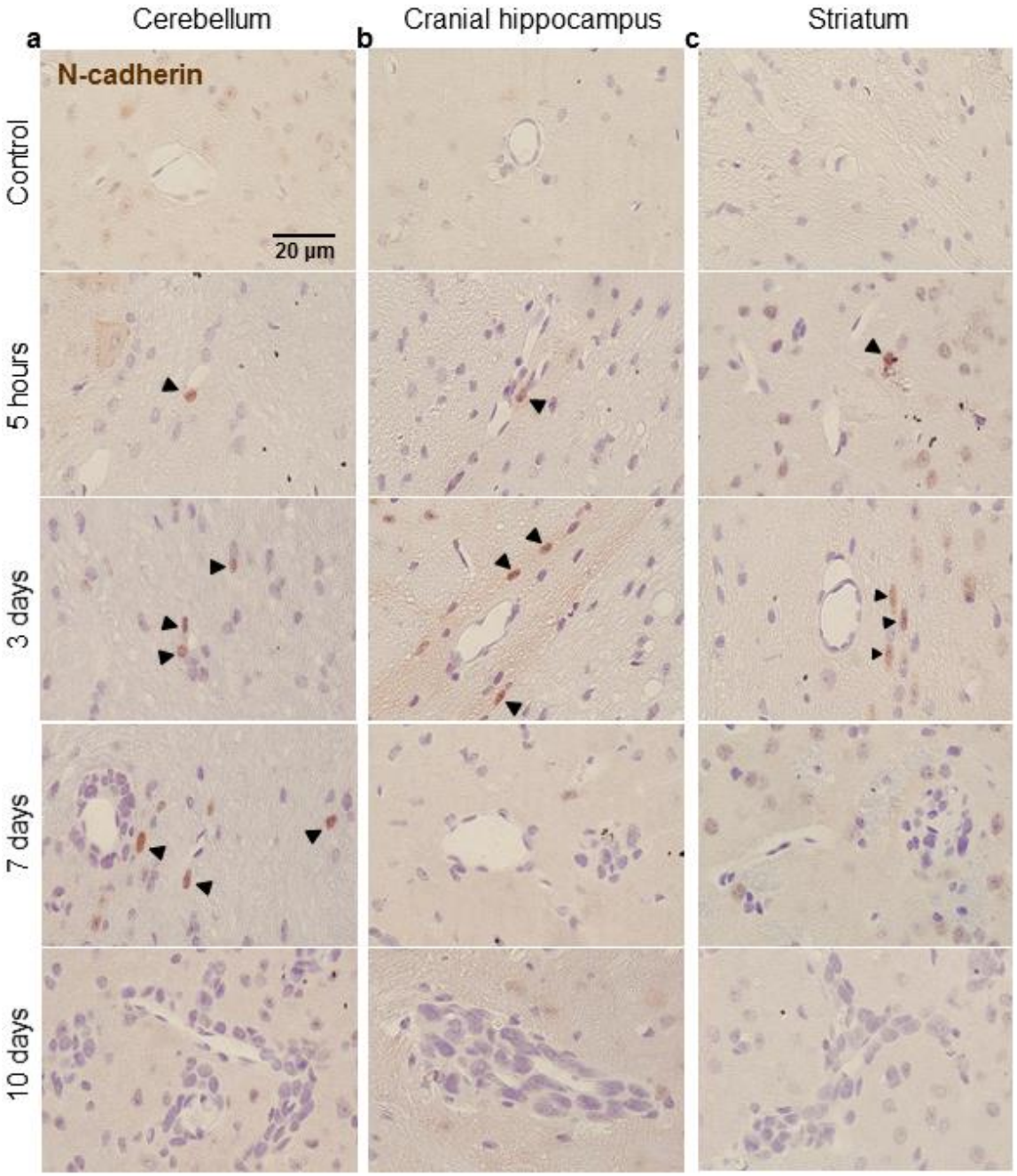


**Fig. 2** Changes in the expression of the mesenchymal marker N-cadherin by breast cancer cells along the process of brain metastasization. Cerebellum, cranial hippocampus, and striatum sections of control mice and of mice at different times post-tumour cell inoculation were processed for immunohistochemical analysis of N-cadherin. Representative immunohistological patterns of N-cadherin-positive cells indicated by arrowheads in cerebellum (a), cranial hippocampus (b), and striatum (c).

blood vessel, and the epithelial phenotype expressed in well-established metastases (Fig. 3). In fact, analysis of vimentin expression by tumour cells of 4T1 injected mice showed an increase over time (Fig. 3a, b, and c). At 5 hours and 3 days, single cancer cells expressing vimentin were observed inside tomato lectin-labelled blood vessels, which do not have a larger diameter than the one of BCCs favouring the extravasation step into the

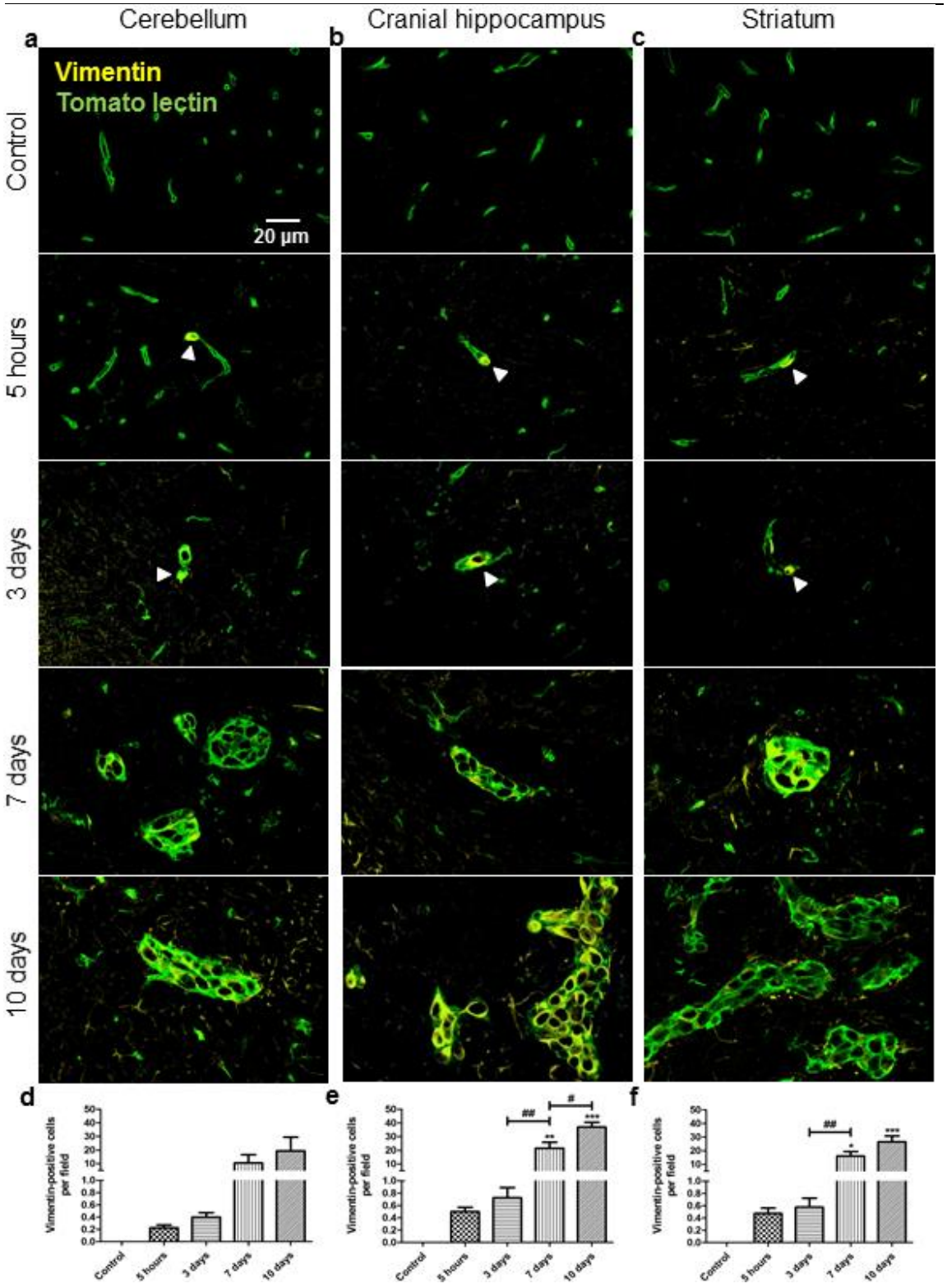


**Fig. 3** Entrance of vimentin-positive breast cancer cells and establishment of tomato lectin-positive macrometastases along the process of brain metastasization. Cerebellum, cranial hippocampus, and striatum sections of control mice and of mice at different times post-tumour cell inoculation were processed for immunofluorescence analysis of the mesenchymal marker vimentin and the epithelial marker tomato lectin. Representative immunohistological patterns of vimentin-positive cells and tomato lectin-positive cells in cerebellum (a), cranial hippocampus (b), and striatum (c), where the arrowheads point to the individual vimentin-positive cells. Semi-quantitative analysis of the vimentin-positive cells per field in cerebellum (d), cranial hippocampus (e), and striatum (f). *P<0.05, **P<0.01, ***P<0.001 vs. control; $^{\#}$P<0.05, $^{\#\#}$P<0.01 between indicated groups.

brain. In line with the results showing that individual N-cadherin-positive cells cross the BBB with a mesenchymal phenotype, the expression of vimentin by malignant cells confirms that BCCs entering the brain present mesenchymal characteristics. Along time, the vimentin continues to be expressed by tumour cells or, at least, by the majority of them, even when these cells were in well-established metastases. Interestingly, tomato lectin expression was also observed in metastases, pointing to the development of epithelial characteristics by malignant cells. Analysis of 4T1 injected mice showed that the number of vimentin-positive cells was remarkably increased from 3 to 7 days post-tumour cell inoculation in the cerebellum (∼26-fold, Fig. 3d), cranial hippocampus (∼30-fold, $P<0.01$, Fig. 3e), and striatum (28-fold, $P<0.01$, Fig. 3f). No further increase in the number of vimentin-positive cells with time (from 7 to 10 days) was observed in the cerebellum and striatum, whereas in cranial hippocampus a ∼2-fold elevation ($P<0.05$, Fig. 3e) was observed in 4T1 inoculated mice. These results showing that BCCs maintain vimentin expression suggest the presence of some mesenchymal features in brain metastases. This result, taken together with the expression of epithelial marker tomato lectin by malignant cells, indicate that BCCs do not undergo a complete MET in the course of brain metastasization.

In order to establish whether tumour cells undergo a complete or a partial MET during brain colonization, we further analysed another epithelial marker, pan Cytokeratin, by double labelling with the same mesenchymal marker, vimentin (Fig. 4, 5). At early stages, we observed that individual cells expressed mainly vimentin or, at least, the expression of vimentin was higher than the pan Cytokeratin expression (Fig. 4a, b, and c). The expression of pan Cytokeratin by tumour cells increased along time, becoming evident when these cells formed well-established metastases. Despite the epithelial phenotype of the malignant cells, BCCs maintain their mesenchymal characteristics, being the largest cellular population observed at 7 and 10 days composed by cells expressing both markers. Interestingly, it was observed that, in some well-established brain metastasis, the malignant cells that were positive only for pan Cytokeratin occupied the core of the metastases, whereas vimentin-positive (whether negative or positive for pan Cytokeratin) were positioned at the periphery of the secondary tumour. For an in-depth analysis of the phenotypical transition, we classified the malignant cells into three groups, according to their phenotype: vimentin-positive/pan Cytokeratin-negative (Fig. 5a), vimentin-positive/pan Cytokeratin-positive (Fig. 5b), and vimentin-negative/pan Cytokeratin-positive (Fig. 5c). The vimentin-positive/pan Cytokeratin-positive cells were

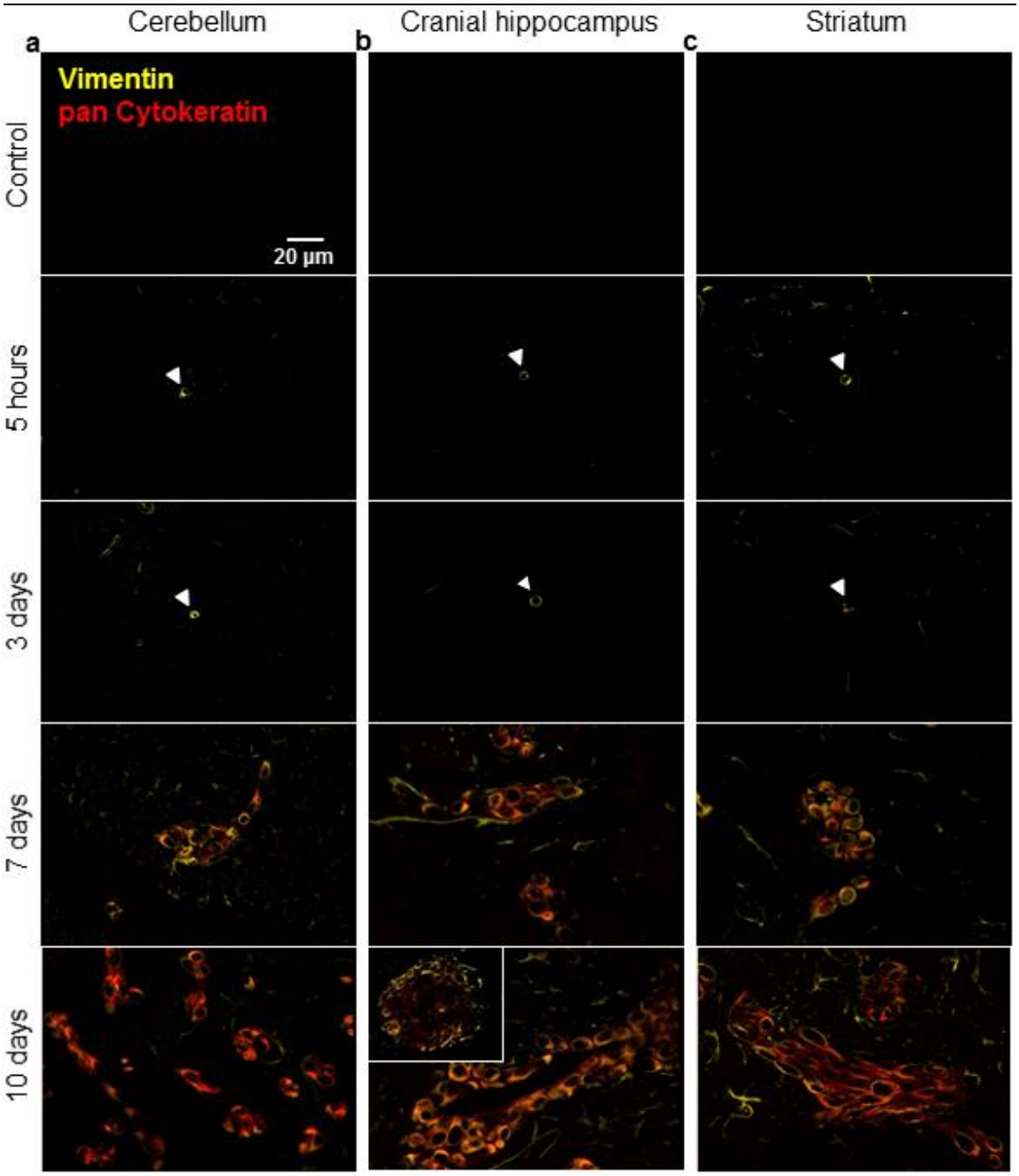


**Fig. 4** Phenotypic changes associated to mesenchymal-epithelial transition of breast cancer cells along the process of brain metastasization. Cerebellum, cranial hippocampus, and striatum sections of control mice and of mice at different times post-tumour cell inoculation were processed for immunofluorescence analysis of the mesenchymal marker vimentin and the epithelial marker pan Cytokeratin. Representative immunohistological patterns of vimentin and pan Cytokeratin double labelling in cerebellum (a), cranial hippocampus (b), and striatum (c), where the arrowheads point to individual vimentin-positive cancer cells in the parenchyma, and the insert shows a well-established metastasis presenting vimentin-negative/pan Cytokeratin-positive cells inside the secondary tumour and vimentin-positive/pan Cytokeratin-negative cells in the periphery of the metastasis.

the most numerous cellular population, reaching ~40 cells per field in cranial hippocampus (Fig. 5h) and in striatum (Fig. 5k) at 10 days, whereas the vimentin-

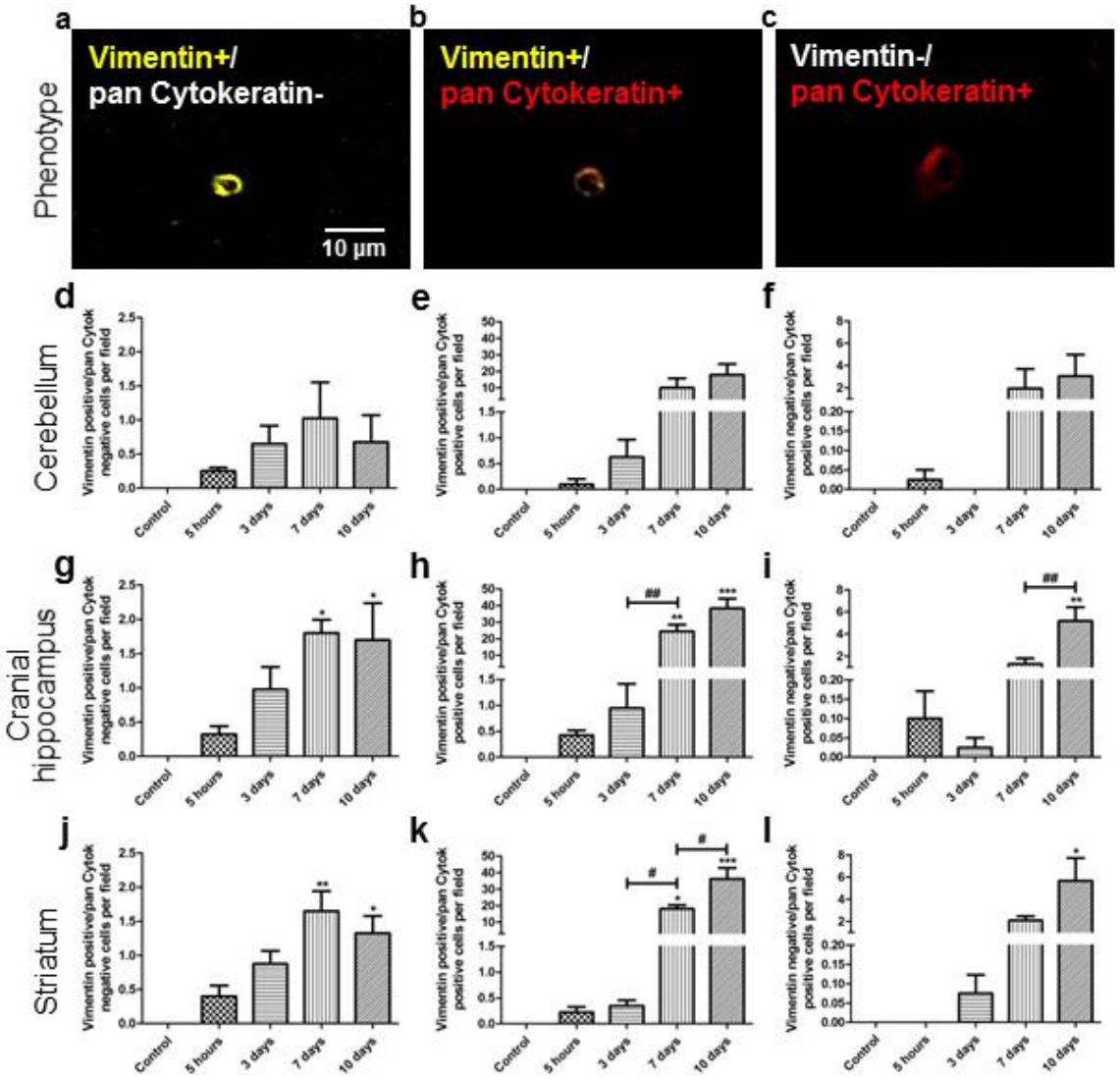


**Fig. 5** Changes in the expression of the mesenchymal marker vimentin and the epithelial marker pan Cytokeratin by breast cancer cells associated to mesenchymal-epithelial transition along the process of brain metastasization. Cerebellum, cranial hippocampus, and striatum sections of control mice and of mice at different times post-tumour cell inoculation were processed for immunofluorescence analysis of vimentin and pan Cytokeratin. Representative immunohistological patterns of vimentin-positive/pan Cytokeratin-negative (a), vimentin-positive/pan Cytokeratin-positive (b), and vimentin-negative/pan Cytokeratin-positive (c) phenotypes. Semi-quantitative analysis of the number of vimentin-positive/pan Cytokeratin-negative cells per field in cerebellum (d), cranial hippocampus (g), and striatum (j). Semi-quantitative analysis of the vimentin-positive/pan Cytokeratin-positive cells per field in cerebellum (e), cranial hippocampus (h), and striatum (k). Semi-quantitative analysis of the vimentin-negative/pan Cytokeratin-positive cells per field in cerebellum (f), cranial hippocampus (i), and striatum (l). *P<0.05, **P<0.01, ***P<0.001 vs. control; $^{\#}$P<0.05, $^{\#\#}$P<0.01 between indicated groups.

positive/pan Cytokeratin-negative cells were the population with the lowest number of malignant cells, never exceeding 2 cells per field in any brain region (Fig. 5d, g, and j). The number of vimentin-positive/pan Cytokeratin-negative cells increased until 7 days

post-tumour cell inoculation in all three brain regions, tending to decrease thereafter in all regions (Fig. 5d, g, and j). Analysis of vimentin-positive/pan Cytokeratin-positive phenotype revealed that during the brain metastasization process there was an abrupt increase of cells expressing this phenotype in all three brain regions from 3 to 7 days (Fig. 5e, h, and k), which was significant in cranial hippocampus (~26-fold, $P<0.01$, Fig. 5h) and striatum (~52-fold, $P<0.05$, Fig. 5k). The number of vimentin-positive/pan Cytokeratin-positive cells continued to increase ~2-fold from 7 to 10 days in all brain regions, varying significantly in striatum ($P<0.05$, Fig. 5k). This increase was also accompanied by an augment of vimentin-negative/pan Cytokeratin-positive cells in all brain regions (Fig. 5f, i, and l), which was significant in cranial hippocampus (~4-fold 7 days vs. 10 days, $P<0.01$, Fig. 5i). In sum, these results show that initially BCCs possess mesenchymal characteristics that may favour the TEM across the BBB and, once inside the brain, tumour cells undergo a total or partial MET to acquire the epithelial phenotype necessary to form metastases.

### 3.3. Metastasization involves a disruption of the BBB by the paracellular pathway

It has been referred that there is an increase of vascularization during the formation of brain metastasis, which is associated to a disruption of intercellular junctions of ECs that compose the blood vessel walls and, in turn, to an increase of BBB permeability (Fan et al. 2011, Avraham et al. 2014). In addition, the TEM of BCCs across the brain endothelium was only described as occurring via paracellular migration (Fan and Fu 2016), a pathway that involved the interaction between the malignant cell and the endothelial junctions. Thus, we investigated the temporal evolution of the BBB permeability based on analysis of the TJ protein claudin-5 immunoreactivity (Fig. 6) and the entrance into the parenchyma of the blood-borne component thrombin (Fig. 7) in the cerebellum, cranial hippocampus, and striatum of control and 4T1 injected mice. Regarding claudin-5, we observed that this TJ protein is expressed at blood vessels (Fig. 6a, b, and c). At early stages of the process of brain metastasization, claudin-5 had a normal distribution forming a continuous or a punctate line at the junction of cell-cell contact, however at 7 days this TJ protein showed an irregular and discontinuous distribution. Curiously, the observation of claudin-5 labelling at 10 days suggested a normal distribution of this TJ protein, similarly to that observed at early stages. Analysis of claudin-5 immunoreactivity of ECs of control and 4T1 injected mice showed a decrease

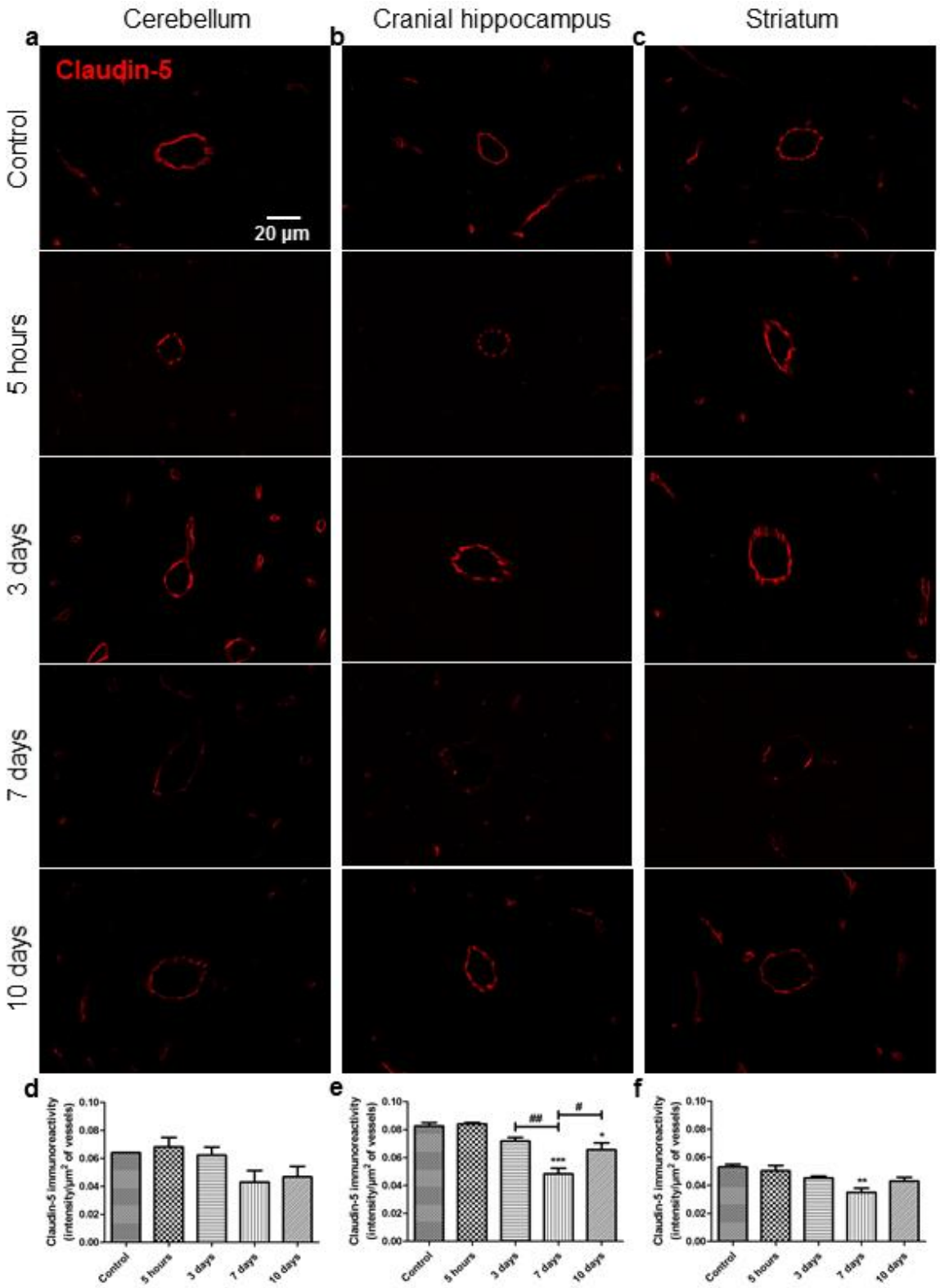


**Fig. 6** Endothelial tight junction protein claudin-5 changes along the process of brain metastasization of breast cancer. Cerebellum, cranial hippocampus, and striatum sections of control mice and of mice at different times post-tumour cell inoculation were processed for immunofluorescence analysis of claudin-5 immunoreactivity. Representative immunohistological patterns of the claudin-5 in cerebellum (a), cranial hippocampus (b), and striatum (c). Semi-quantitative analysis of the claudin-5 immunoreactivity in endothelial cells junctions per field in cerebellum (d), cranial hippocampus (e), and striatum (f). *P<0.05, **P<0.01, ***P<0.001 vs. control; $^{\#}$P<0.05, $^{\#\#}$P<0.01 between indicated groups.

over time, and that this decrease was significant at 7 days in both cranial hippocampus (42% vs. control, $P<0.001$, Fig. 6e) and striatum (34% vs. control, $P<0.01$, Fig. 6f). At this timepoint, the cerebellum also showed a 33% decrease of claudin-5 immunoreactivity as compared with the control (Fig. 6d), even though not significant. Remarkably, a 27% increase of claudin-5 immunoreactivity between 7 and 10 days was observed in cranial hippocampus ($P<0.05$, Fig. 6e), but not reaching the control values. Although not significant for the other brain regions, this increase was also observed in cerebellum (8%, from 7 to 10 days) and in striatum (19%, from 7 to 10 days). Thus, these results show that the migration of BCCs into the brain is accompanied by a disruption of intercellular junctions of the ECs that compose the BBB, suggesting an hyperpermeability by the paracellular route that may be associated with the migration of malignant cells across BBB endothelium. Moreover, these results also suggest that the BBB has the ability to repair, at least partially, its barrier properties after the passage of metastatic tumour cells into the brain.

In addition to claudin-5 immunoreactivity, we investigated if the disruption of endothelial junctions is accompanied by the entrance into the brain parenchyma of thrombin, used as an indicator of BBB disruption (Janota et al. 2015). To this end, we measured the total number of thrombin deposits per section (Fig. 7), which were normally localized near blood vessels and occasionally near metastasis. Small thrombin deposits were already evident at 3 days post-tumour cell injection ($P<0.05$ in striatum, Fig. 7f), in line (Fig. 7a, b, and c). An increase of the dimensions of thrombin deposits was observed in all three brain regions of 4T1 injected mice from 3 to 7 days, further increasing until 10 days. Similarly to deposits dimensions, an increase of the number of thrombin deposits was also detected in all three brain regions along the process of brain metastasization (Fig. 7d, e, and f). Interestingly, the higher number of thrombin deposits was detected in the cranial hippocampus (Fig. 7e), which was the region presenting the lower claudin-5 immunoreactivity of ECs between 5 hours and 7 days, which suggests a higher permeability of the BBB (Fig. 6e). Remarkably, the highest number of thrombin deposits at 10 days did not correspond to the lower claudin-5 immunoreactivity at 7 days. Thus, the results of claudin-5 immunoreactivity taken together with the results of thrombin suggest an increase of BBB permeability along the brain metastasization process. Moreover, they suggest that there is some recover of the TJ protein claudin-5 integrity, which is not enough to prevent the entrance of the blood-borne component thrombin at later stages.

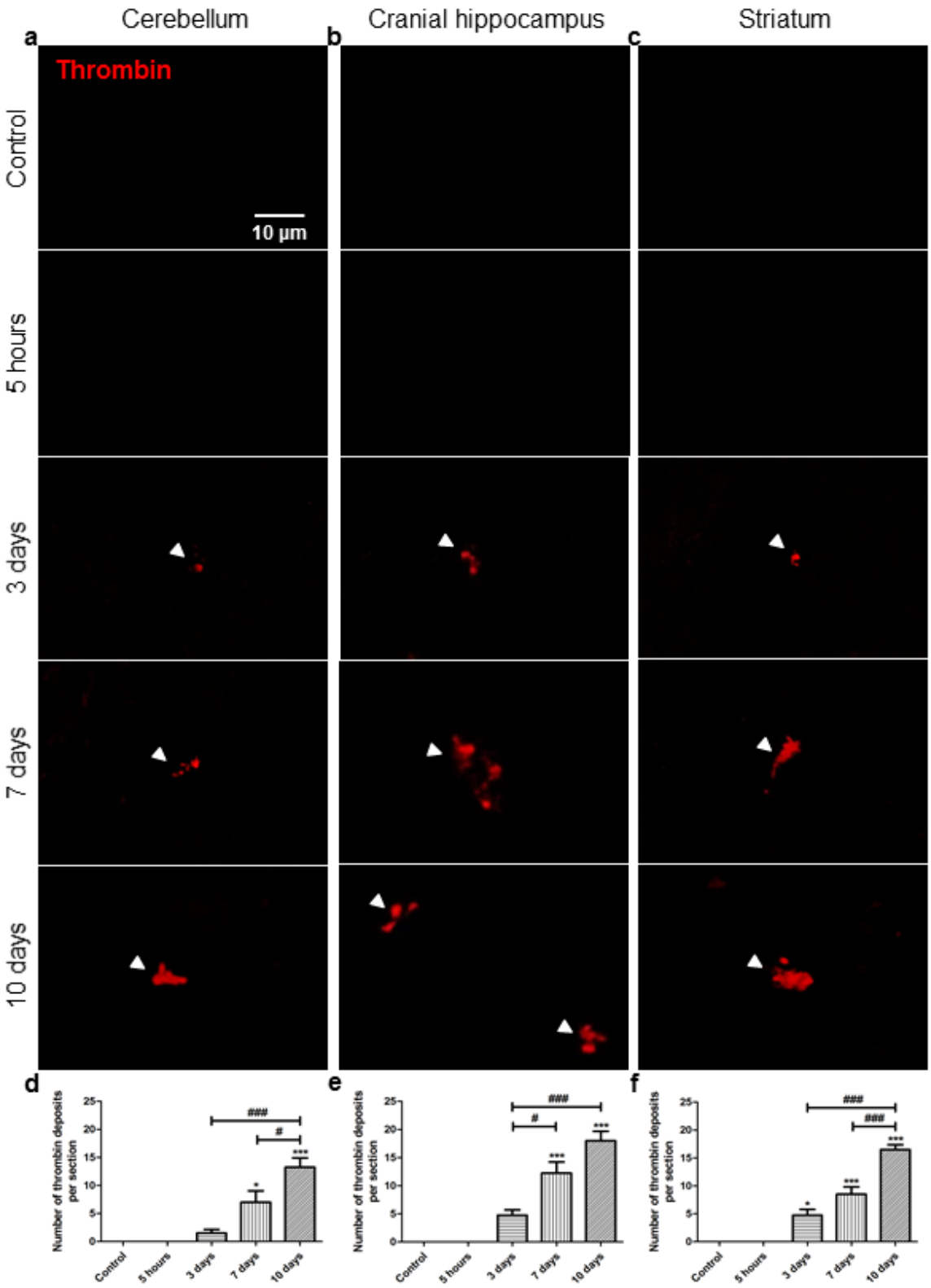


**Fig. 7** Entrance of the blood-borne component thrombin into the brain parenchyma along the process of brain metastasization of breast cancer. Cerebellum, cranial hippocampus, and striatum sections of control mice and of mice at different times post-tumour cell inoculation were processed for immunofluorescence analysis of the blood-borne component thrombin. Representative immunohistological patterns of thrombin in cerebellum (a), cranial hippocampus (b), and striatum (c), where the arrowheads point to thrombin deposits. Semi-quantitative analysis of total number of thrombin deposits per section in cerebellum (d), cranial hippocampus (e), and striatum (f). *P<0.05, ***P<0.001 vs. control; $^{\#}$P<0.05, $^{\#\#\#}$P<0.001 between indicated groups.

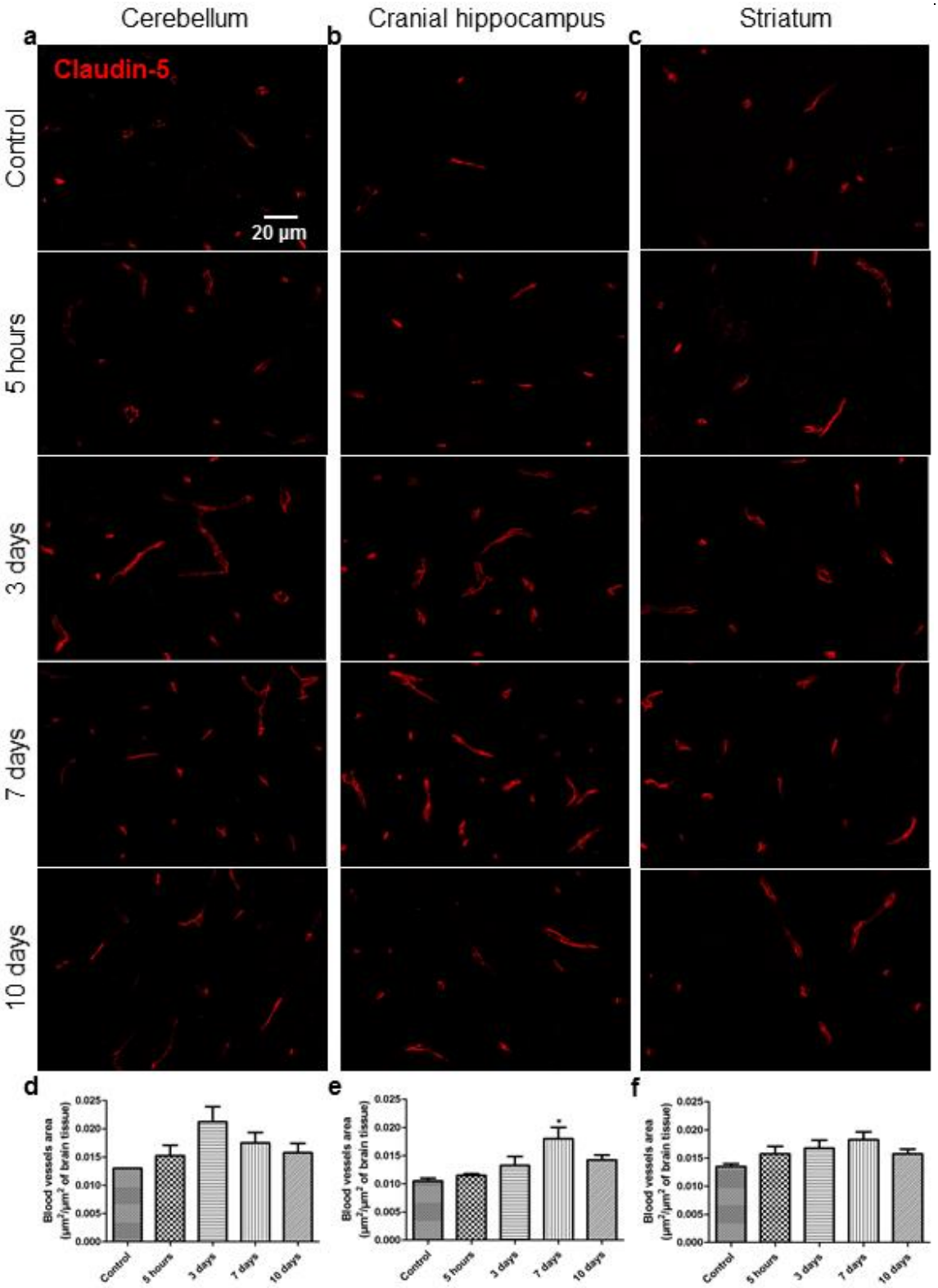


**Fig. 8** Microvascular density changes along the process of brain metastasization of breast cancer. Cerebellum, cranial hippocampus, and striatum sections of control mice and of mice at different times post-tumour cell inoculation were processed for immunofluorescence analysis of endothelial protein of tight junctions claudin-5. Representative immunohistological patterns of claudin-5 in cerebellum (a), cranial hippocampus (b), and striatum (c). Semi-quantitative analysis of the area of blood vessels per brain area in cerebellum (d), cranial hippocampus (e), and striatum (f). *P<0.05 vs. control.

### 3.4. Formation of brain metastasis requires increased vascularization

Previous studies have associated the formation of primary and secondary tumours to an increased vascularization (Monsky et al. 2002, Tang et al. 2005, Avraham et al. 2014), which prompted us to investigate the temporal evolution of microvascular density in the present study. Cluster of differentiation (CD)31, CD34, and tomato lectin are three widely used endothelial markers of microvessels (Bell et al. 2010). However, we did not obtain any specific labelling in our tissue sections for CD31 and CD34, whereas tomato lectin also labels epithelial cells and, thus, malignant cells with an epithelial phenotype, as shown in Figure 3. As an alternative, we analysed claudin-5-labeled sections to determine the density occupied by the microvasculature in the cerebellum, cranial hippocampus, and striatum of control and 4T1 injected mice (Fig. 8). Observation of claudin-5 stained sections of control mice suggested that the cerebellum and the striatum were more vascularized than the cranial hippocampus (Fig. 8a, b, and c). The vascularization augmented in all three brain regions along time in 4T1 injected mice. However, this increase was accompanied by a slight decrease of microvascular density from 7 to 10 days. The microvessel density determined in control mice was 0.014 $\mu m^2/\mu m^2$ in striatum (Fig. 8f), 0.013 $\mu m^2/\mu m^2$ in the cerebellum (Fig. 8d), and 0.010 $\mu m^2/\mu m^2$ in cranial hippocampus ($P<0.05$ vs. striatum; Fig. 8e). Regarding 4T1 injected mice, it was not observed a significant increase of microvessel density in cerebellum and striatum along time compared to control mice, while in cranial hippocampus a significant increase of ~2-fold between the control and 7 days post-tumour cell inoculation was observed ($P<0.05$, Fig. 8e). Thus, the process of brain colonization by BCCs appears to be associated with hypervascularization in cranial hippocampus.

### 3.5. Malignant cells express PDGF-B

We wanted to know if BCCs colonizing the brain express the growth factor PDGF-B that was shown to be expressed by malignant cells associated to lymphatic and bone metastasization (Lev et al. 2005, Schito et al. 2012). For that purpose, we analysed PDGF-B-positive cells along time (Fig. 9). Curiously, in all three brain regions, this ligand was predominantly expressed by tumour cells normally located near a blood vessel, rather than by ECs (Fig. 9a, b, and c). Analysis of PDGF-B-positive cells in cerebellum (Fig. 9d), cranial hippocampus (Fig. 9e), and striatum (Fig. 9f) showed that the number of cells expressing this ligand was low at 5 hours, not showing a significant alteration of the number of positive cells compared to control. PDGF-B-positive cells

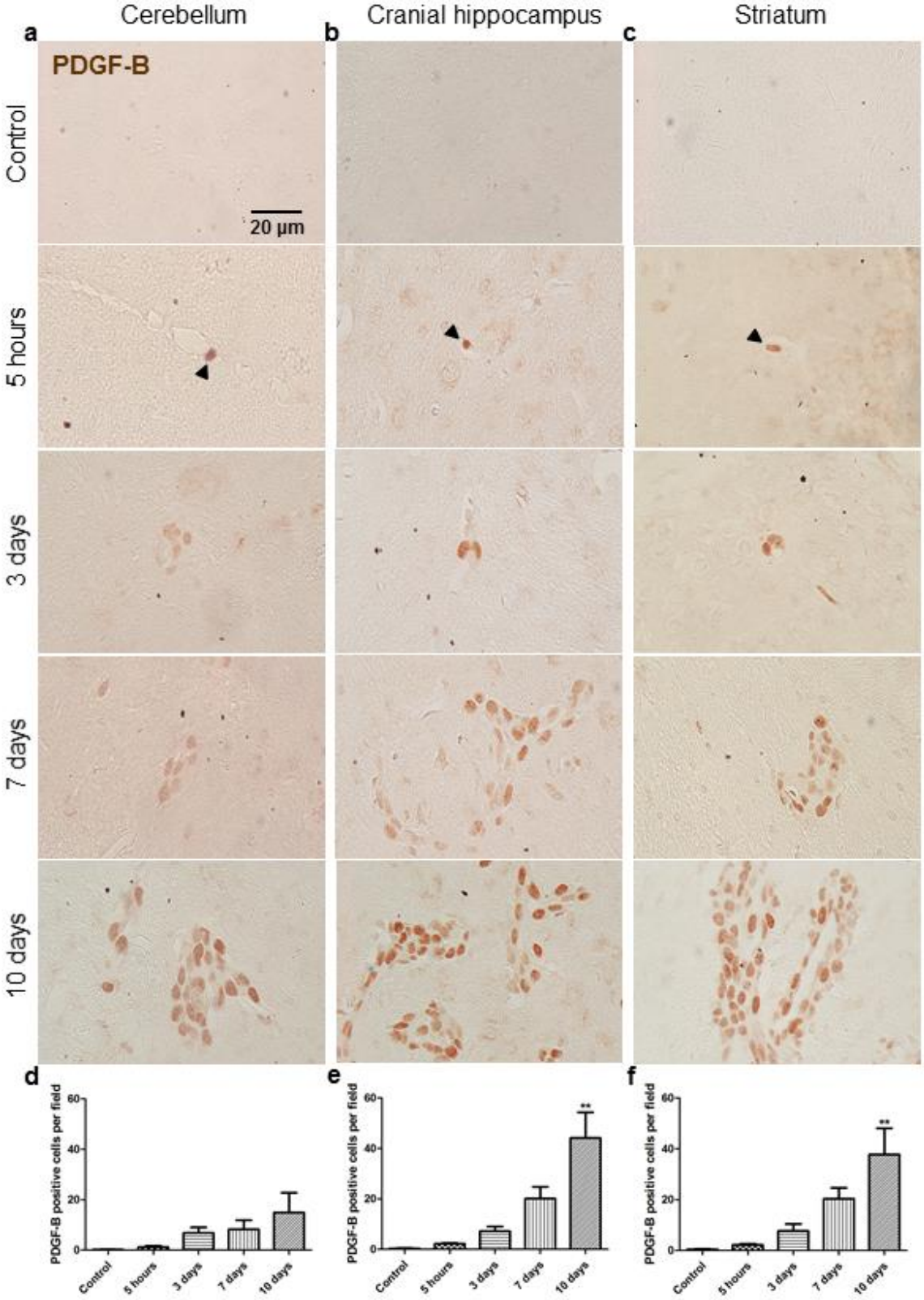


**Fig. 9** Changes in the expression of the platelet-derived growth factor B (PDGF-B) by breast cancer cells along the process of brain metastasization. Cerebellum, cranial hippocampus, and striatum sections of control mice and of mice at different times post-tumour cell inoculation were processed for immunohistochemical analysis of PDGF-B. Representative immunohistological patterns of PDGF-B in cerebellum (a), cranial hippocampus (b), and striatum (c), where the arrowheads point to the individual cells that express this growth factor. Semi-quantitative analysis of PDGF-B-positive cells per field in cerebellum (d), cranial hippocampus (e), and striatum (f). **P<0.01 vs. control.

were already evident at 3 days, reaching a fold around 100 or higher of positive cells at 10 days post-tumour cell inoculation in all brain regions. Thus, these results show that the expression of PDGF-B by tumour cells increases from the timepoint where entry of these cells into the brain occurs to the formation of well-established brain metastasis.

### 3.6. Malignant cells express CXCR4

Previous work suggested that the arrival of viable cancer cells to the brain can result from a trapping of circulating tumour cells (CTCs) in small vessels of this secondary organ driven by organ-derived chemoattractants, being one of the most studied pairs the CXCR4 and its ligand CXCL12 (Hujanen and Terranova 1985, Salmaggi et al. 2014). Thus, it seems that there is an important link between CXCR4/CXCL12 axis and the homing of carcinoma cells into the brain, contributing to metastatic progression. In order to understand the temporal evolution of entrance of CXCR4-positive tumour cells into the brain along the metastasization of this secondary organ, we measured the number of cells expressing the receptor per field (Fig. 10). Interestingly, CXCR4-positive cells were only observed as individual cells and not in well-established metastases (Fig. 10a, b, and c). Analysis of 4T1 injected mice showed that the number of CXCR4-positive cells was higher at 3 days than any other timepoint (Fig. 10d, e, and f). This result is in line with the analysis of vimentin-positive cells at 5 hours and 3 days (Fig. 3), since either for CXCR4 or for vimentin, an increase of malignant cells extravasating at these two timepoints was already observed. In addition, it was interesting to observe a slight decrease of CXCR4-positive cells at 7 and 10 days compared to 3 days, suggesting a continuous entrance of malignant cells along the process of brain metastasization.

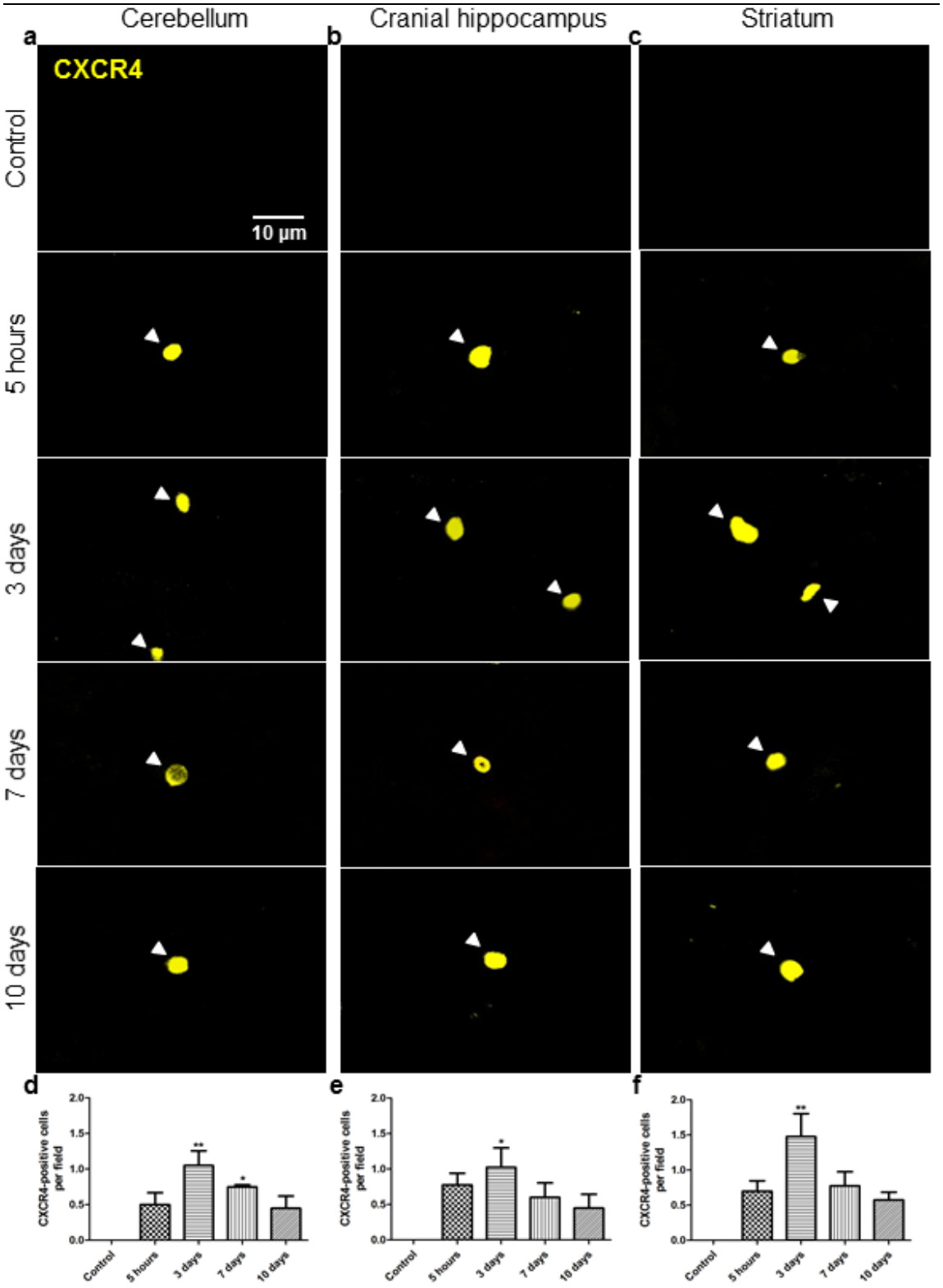


**Fig. 10** Changes in the expression of cysteine-X amino acid-cysteine receptor 4 (CXCR4) by breast cancer cells along the process of brain metastasization. Cerebellum, cranial hippocampus, and striatum sections of control mice and of mice at different times post-tumour cell inoculation were processed for immunofluorescence analysis of CXCR4. Representative immunohistological patterns of CXCR4 in cerebellum (a), cranial hippocampus (b), and striatum (c), where the arrowheads point to individual cells that express this receptor. Semi-quantitative analysis of the number of CXCR4-positive cells per field in cerebellum (d), cranial hippocampus (e), and striatum (f). *P<0.05, **P<0.01 vs. control.

## 4. Discussion

Compared to other 'target organs' of BCCs, such as the bone, the mechanisms responsible for brain metastasis formation remain poorly understood and substantially less investigated. The brain is a very peculiar organ protected by the BBB, which represents the tightest endothelial barrier in the organism (Cardoso et al. 2010). However, this barrier allows the transmigration of malignant cells into the brain (Molnár et al. 2016), and consequent formation of metastases in this secondary organ. It is nowadays believed that the interaction between the metastatic cells and BMVECs plays an important role in brain metastasization of BC, namely, in promoting the attraction of cancer cells into the brain vasculature, their transmigration, and even tumour-associated vascular development (Müller et al. 2001, Zhao et al. 2011, Fan and Fu 2016). Prior to TEM across the BBB into the brain parenchyma, CTCs adhere to the brain endothelium (Kienast et al. 2010, Stoletov et al. 2010). However, the features of malignant cells present during the TEM and along the colonization of this secondary organ are still unclear. In addition, BBB integrity is intimately associated with the route used by malignant cells to transpose the brain endothelium, and its role in the process of brain metastasization is largely unexplored. The only route described in the brain is the paracellular pathway, in which malignant cells must interact with the intercellular junctions of two BMVECs to enter the brain (Fan and Fu 2016, Vandenhaute et al. 2016). Nonetheless, it is unkown whether the passage of tumour cells keeps the BBB intact or whether BBB has the ability to repair its barrier properties after the passage of metastatic cancer cells into the brain. Thus, we aimed to understand and characterize the process of brain metastases formation, by examining the temporal profile of BCCs metastasization to the brain, metastasizing cells phenotype, BBB integrity, vascular density, and signalling molecules involved in attraction and proliferation of malignant cells during disease progression.

To induce the formation of brain metastases of BC, we used the 4T1 cells, which is one of the most aggressive metastatic murine BCC lines able to colonize the brain (Tao et al. 2008, Gao et al. 2011). The inoculation of tumour cells into the right common carotid artery not only reflects the haematogenous dissemination (Saito et al. 2008), one route used by cancer cells to escape from the primary tumour, but also directs the malignant cells to the brain, allowing the formation of metastases solely in this secondary organ (Zhang et al. 2008, Lorger and Felding-Habermann 2010). As brain metastasization progressed, we observed metastasis within the parenchyma of cerebellum, cranial

hippocampus, and striatum that gradually occupy an increasing tumour area. Curiously, cerebella of 4T1 injected mice developed brain metastases with smaller dimensions compared to those formed in cranial hippocampi, and striata. This difference may be explained by the anatomical distance between the right common carotid artery and the cerebellum. Thus, the majority of malignant cells get trapped in brain regions closer to the inoculation site, such as cranial hippocampus and striatum, and the cells that do not get trapped take longer to reach the cerebellum and are a minority. Based on the temporal and special profile of establishment of brain metastasis, the majority of cancer cells arrest and extravasate into cranial hippocampus and striatum, and a minority into cerebellum. In addition, the number of 4T1 cells injected in mice is also a feature that allows the rapid intracranial growth of tumour cells, leading to death of some animals even after 10 to 14 days post-tumour cell inoculation, as described previously (Lorger and Felding-Habermann 2010).

We analysed the time-course of cancer cell extravasation into the brain parenchyma and characterized the phenotype expressed by the malignant cells during the exit from bloodstream and along the brain metastatic process. A previous *in vivo* study, using the same method of cancer cell inoculation and bioluminescence imaging, showed that 4T1 cells were readily detected throughout the brain parenchyma in very early stages of brain metastasization process, being detected inside the brain even 1 day post-tumour cell inoculation (Lorger and Felding-Habermann 2010). Furthermore, the extravasation process has been described as occurring after tumour cells arrest in small capillaries (Lorger and Felding-Habermann 2010, Stoletov et al. 2010). In line with these reports, here we show that BCCs are trapped in capillaries with a similar diameter to that presented by the carcinoma cells even 5 hours post-tumour cell injection. These cells present mesenchymal characteristics expressing both N-cadherin and vimentin proteins, whereby the fusiform morphology favours the penetration of brain endothelium by cancer cells. The upregulation of these mesenchymal markers expression is associated to tumour progression and increase of cancer cell invasiveness (Agiostratidou et al. 2007, Calaf et al. 2014). N-cadherin is a transmembrane adhesion molecule normally found in neural, mesenchymal and connective cells and is expressed by both BCCs and BMVECs (Harzheim et al. 2004, Strell and Entschladen 2008), allowing the attachment of the malignant cells to the endothelial ones. Furthermore, we found that once in well-established metastasis, malignant cells do not express N-cadherin, suggesting that these cells downregulate the expression of this adhesion molecule, and probably up-regulate

the expression of other molecules, such as endothelial-cadherin, responsible for interactions between epithelial cells, as previous described (Chao et al. 2012). On the other hand, vimentin is an intermediate filament protein involved in cytoskeleton rearrangement, which in turn contributes to cell motility. Moreover, vimentin is involved in the regulation of the expression of Slug, an EMT-related transcription factor, which enhances cancer malignancy (Herrmann et al. 1996, Qiao et al. 2015). In addition to vimentin-positive cells localized inside or near blood vessels, malignant cells in well-established metastases also express this mesenchymal marker. Thus, this result suggests that the BCCs undergo a partial MET.

Based on previous observations of MET in brain metastasis developed by BCCs (Chao et al. 2012, Yoshida et al. 2014), we expanded our analysis to include other epithelial marker, pan Cytokeratin, to discern whether a full or partial MET occurs. At early timepoints, the majority of the cancer cells express mainly vimentin. This result showed that initially BCCs possess mesenchymal characteristics that may favour the TEM across the BBB. Over time, it is not surprising to observe an increase of pan Cytokeratin expression in tumour cells, revealing that once inside the brain malignant cells acquire the epithelial characteristics to favour the formation of well-established metastases. Curiously, at later stages of the brain colonization, the majority of BCCs express both mesenchymal and epithelial markers, suggesting that the malignant cells maintain their phenotypic plasticity by undergoing a partial MET, as previously described using BC samples from patients with brain metastases (Chao et al. 2012). These cells, as the ones that only express vimentin, have the ability to migrate to other areas of the brain, where they can establish new secondary tumours. The mesenchymal phenotype expressed by BCCs was also suggested as being a tumour protective mechanism against inflammatory or chemotherapeutic insult (Chao et al. 2012). It is also noteworthy to mention the observation of metastases growing around or near blood vessels. Once BBB is crossed, carcinoma cells are able to spread along brain capillaries, initiating brain metastatic lesion formation in association with vasculature (Carbonell et al. 2009, Stoletov et al. 2013, Valiente et al. 2014). This location of cancer cells favours the proliferation not only because the cells have an optimal oxygen supply and access to nutrients, but also because the BBB provides protection against antitumour immunity and chemotherapeutics drugs (Carbonell et al. 2009, Alkins et al. 2013, Yoshida et al. 2014).

The BBB impermeability is most pronounced for molecules greater than 800 Da and the TJ proteins, such as claudin-5, play a crucial role in regulating endothelial

permeability (Nitta et al. 2003, Ohtsuki et al. 2007). However, one of the key features of brain metastasization is the paracellular TEM of BCCs across the BBB. This transmigration route involves the BBB breakdown via disruption of endothelial junctions, which in turn causes an increase of BBB permeability (Lee et al. 2003, Avraham et al. 2014, Rodriguez et al. 2014). Our results corroborate these studies by showing a decrease of claudin-5 immunoreactivity and an increase of thrombin deposits in brain parenchyma. Of note in our study, the detection of the higher number of blood-borne component deposits in the parenchyma does not happen at the same timepoint of the lower value of claudin-5 immunoreactivity. This could be due to the continuous entrance of thrombin once the cancer cell causes an impairment in BBB enough for a 37 kDa molecule to be able to cross the brain endothelium. The continuous thrombin passage is confirmed by the number of thrombin deposits that progressively augment in brain parenchyma. The increase of this protein is an early event in mice injected with 4T1, suggesting that BBB disruption occurs as a result of the early passage of BCCs across the brain endothelium. Thence, the entire extravasation process of a 4T1 cancer cell must occur in less than 3 days since at this timepoint thrombin is already detected in brain parenchyma. Thus, contrarily to the duration of TEM estimated *in vivo* (3 to 5 days) (Lorger and Felding-Habermann 2010), this result demonstrated that extravasation of triple-negative cells, such as 4T1 cancer cells, occurs in shorter period than the estimated one. In addition, the proliferation of cancer cells and formation of macrometastases around blood vessels can be other explanation for the BBB impairment. Interestingly, at the last timepoint of our study, there is an increase of claudin-5 immunoreactivity, suggesting that the BBB possesses a repair and/or reorganizing capacity that allows the reestablishment of endothelial junctions.

We analysed how brain microvasculature density through measurement of the area of blood vessels visualized by claudin-5 labelling, since previous studies described an increased vascularization during the formation of secondary tumours (Monsky et al. 2002, Avraham et al. 2014). Herein, we further show that the hypervascularization occurs only in cranial hippocampus. This hypervascularization at a later stage may be related to the fact that initially cancer cells obtain an adequate blood supply by vascular co-option (Leenders et al. 2002, Bugyik et al. 2011), not requiring the formation of new blood vessels. Vascular co-option and angiogenesis have distinct contributions during the initiation of micrometastases, depending not only on the cell numbers in the microtumour, but also on blood supply of local microenvironment (Zhao et al. 2011). In addition, we

hypothesize that the heterogeneity of brain microvasculature might also explain the hypervascularization only observed in cranial hippocampus. Although a characterization of the differences regarding brain microvasculature within the different brain regions under physiological conditions has not been performed, our results of control mice suggest that the cranial hippocampus has lower microvessel density compared to striatum. Thus, the lower microvessel density and the higher tumour area in cranial hippocampus may favour the formation of new vessels, leading to hypervascularization in this brain region.

To the best of our knowledge, this study is the first one to provide quantitative data of the temporal and spatial expression of PDGF-B by BCCs during the formation of brain metastases. PDGF-B is a growth factor involved in regulation of cellular processes such as migration, proliferation, and differentiation (Lindberg and Holland 2012). Regarding BC, both benign and malignant breast cells have been described as expressing PDGF-B in lymphatic and bone metastases (Coltrera et al. 1995, Lev et al. 2005, Schito et al. 2012). In addition, the expression of this growth factor in tumour cells can be directly induced by the transcription factor LIM homeobox gene 2 (Lhx2), which is upregulated during EMT in BCCs (Kuzmanov et al. 2014). The Lhx2 has a dual role during EMT and tumour progression: by inducing the expression of PDGF-B, Lhx2 promotes an autocrine PDGF-B/PDGF receptor beta (PDGFR-β) loop required for cell migration, invasion and metastatic dissemination and paracrine PDGF-B/PDGFR-β signalling to support blood vessel functionality and, thus, tumour growth (Kuzmanov et al. 2014). Regarding the nervous tissue, PDGF signalling has been only associated to glioma cells that are dependent of this growth factor to maintain proliferative capacity (Uhrbom et al. 2004). In addition, a recent study demonstrated that highly invasive glioma initiating cells express high levels of PDGF-B, and the PDGF signalling may have a role in the transformation of host glial cells that have a high expression of PDGF receptors (Chen et al. 2015). Similarly to glioma cells, our results show that BCCs also express the growth factor PDGF-B that may favour their own proliferation and subsequent formation of brain metastases. Thus, these data point to PDGF-B as a player in the development of brain metastases by BCCs and a new therapeutic target for future treatment.

Lastly, we studied the temporal evolution of CXCR4 expression by cancer cells in brain parenchyma. Our results reveal that extravasating BCCs express the receptor CXCR4, which is in line with the association of CXCR4 to an increased migratory and invasive potential required during the haematogenous journey to the brain and during the

penetration across the brain endothelium (Cronin et al. 2010). The observation of CXCR4-positive cells in brain parenchyma along the metastatic process suggests a continuous entrance of these cells even 10 days post-tumour cell injection. Interestingly, brain metastases formed by 4T1 cells do not express CXCR4. This result suggests that the downregulation of CXCR4 expression is associated to decrease migratory and invasive capacity of the malignant cells. However, not only the expression of vimentin contradicts the loss of this capacity, but also CXCR4 expression is upregulated by hypoxia, which has been associated with brain metastases of BC (Cronin et al. 2010, Saha et al. 2011).

In conclusion, our findings suggest that cancer cells induce a variety of BBB and brain parenchyma alterations, as schematically depicted in Figure 11, that can be target in order to prevent the brain metastasis by BCCs. Therefore, BBB stabilizing drugs or drugs that contribute to vascular density maintenance may have beneficial effects in metastasization of BC by preventing the entrance and dissemination of tumour cells into the brain. In addition, our results suggest that PDGF-B plays an important role in cancer cell proliferation and targeting this growth factor or its receptors may be useful in treatment of secondary tumours. Thus, this study provides new insights into the mechanisms of brain metastatic cancer.

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# Chapter III

## Final considerations

## Concluding remarks and perspectives

Metastasis of breast cancer (BC) to the central nervous system (CNS) continues to be a great challenge. Despite the considerable improvement made in BC treatment over the past decades, the brain metastasization remains an unsolved problem. Thus, it is important to prevent the entrance of malignant cells into the brain and the establishment of metastasis in this secondary organ. The CNS is protected against neurotoxicity and entry of pathogens and cells by the blood-brain barrier (BBB), a highly selective barrier that prevents the delivery of the current chemotherapeutic agents, but is not able to block the entrance of cancer cells into the brain. In fact, the BBB has a pivotal role along the process of brain metastasization, namely during the transendothelial migration (TEM) via paracellular route, making the BBB modulation as a therapeutic target in metastatic BC. Hence, there could be two approaches according to the stage of BC: for early stages or metastatic spread to organs such as the bones, lungs or liver, the sealing of BBB must prevent the entrance of malignant cells, and for metastatic stages when the brain metastases are already formed, the improvement of BBB permeability would allow the entrance of chemotherapeutic agents. It is also important to understand the mechanisms that lead to BBB disruption and whether this disruption is a reversible event. Notably, TJs have a crucial role in BBB integrity, and their modulation may be a promising approach to prevent the extravasation of tumour cells into CNS. In addition, there are evidences that the neurovascular unit cells are involved in TJ formation and modulation, being also important to study the interactions between these cells and the mentioned junctions. Further research in this field may reveal more therapeutic opportunities to prevent brain metastasis formation associated with BBB breakdown.

Although there is only evidence that malignant cells use the paracellular route to migrate into the brain, the hypothesis that cancer cells may also migrate through the endothelial barrier by transcellular TEM remain to be established. Thus, it could be interesting to find out if the malignant cells also cross the brain endothelium via transcellular route. Since the myosin light chain kinase (MLCK) activation and contraction of cytoskeletal myosin in endothelial cells (ECs) have been described to be involved in transcellular pathway during intravasation into blood vessels, the study of these proteins could be a starting point. Then, the study could evolve to the establishment of the expression of proteins involved in vesicular transport, such as caveolin-1, and the determination of basement membrane and extracellular matrix proteins expression. Thus,

this study could provide clues about the transcellular migration of BC cells (BCCs) across the brain endothelium.

In the work here presented, it was shown that the formation of brain metastases could occur through the continuous entrance of malignant cells into the brain and involve the expression of growth factors that favour the tumour cell proliferation. Firstly, to complete the present study and confirm whether the platelet-derived growth factor B (PDGF-B)-positive cells are proliferating, it would be interesting to perform a double labelling with PDGF-B and Ki-67, a proliferation marker. Secondly, the downregulation of PDGF-B expression in BCCs could be a promising therapeutic target. Thirdly, since the PDGF-B/platelet-derived growth factor receptor beta (PDGFR-β) signalling is involved in the cross-talk between ECs and pericytes and these latter cells are the neurovascular unit cells less studied in brain metastasization, it would be interesting to assess whether the pericytes change their localization in blood vessel wall to allow the passage of malignant cells or the passage of malignant cells induces the reduction the number of pericytes around the blood vessels.

Despite the progress in the current knowledge about the mechanisms behind the process of brain metastasization, there are still major gaps in our understanding that must be filled. So, a better comprehension of the time-course and cellular and molecular players that are involved in the process of brain metastasization of BC may have a huge impact for the identification of novel targets against the formation of secondary tumours. Hopefully, clarification of these issues will allow to timely establish preventive approaches to avoid the occurrence of brain metastasis and delay the cancer progression.